Chapter 1

Selective Cleavage of Tyrosyl Peptide Bonds
1.1 INTRODUCTION
Recent years have witnessed the growth of a new field of inquiry, in which attempts are made to correlate precisely the unique physical, chemical and functional properties of a given protein with particular aspects of its structure. One approach to elucidating structure-function relationships has been to study effects on biological activity of specific covalent alterations in the native structure of the protein in question. A rapid expansion in our knowledge of protein sequences, which began in early 1960's and which resulted directly from the development and application to protein chemistry of high resolution analytical methods, generated a corresponding growth in the area of chemical modification studies. Considerable attention is currently focussed on the development of specific reagents for selective modification of amino acid residues. Studies designed to alter the course of proteolytic cleavage and to facilitate or actually bring about the specific chemical cleavage of the protein have emerged as a fascinating and fruitful area of research.
Polypeptide cleavage is the first stage in the classical method of primary structure analysis of proteins. Since fragmentation of the protein molecule to smaller peptides is probably the key reaction in the degradative processes, the search for specific reagents for the selective cleavage of various peptide bonds and standardization of existing techniques are of prime importance. Careful consideration must be given to questions regarding side reactions and specificity of such reagents under various experimental conditions. Many of the proteolytic enzymes currently employed are not specific in their action. Further, many parts of the protein molecule in the native state may not be accessible to the action of such enzymes. Thus, there is an acute shortage of ideal methods for specific peptide cleavage.

Choice of suitable cleavage methods is dictated by the need to obtain comparatively few fragments in good yields. Amino acid residues that occur relatively infrequently in proteins are therefore the preferable sites for attack. Selective cleavage of only a few peptide bonds generally simplifies peptide fractionation because a small number of peptides are
present in the digestion mixture. Selective cleavage also simplifies peptide alignment because fewer overlapping peptide fragments are obtained. The recent advances in the automated sequence analysis of proteins have also emphasized the need for the development of newer cleavage reactions for generation of specific peptide fragments. The methodology of amino acid sequence analysis has received specific benefits from organic chemistry in the form of procedures for selective cleavage of peptide bonds.³

The chemical reactivity of proteins, arises from the properties of side chain amino acids. Selective chemical cleavage is brought about by neighbouring group effects, relying upon a distinguishing reactivity of a particular amino acid side chain or of a chain terminus. Selective cleavage makes use of intramolecular assistance, but departs from conventional nucleophilic hydrolytic agents in favour of electrophilic agents or groups. The roles in this case have thus been reversed. In hydrolysis, the amide carbon (A) serves as an attractant (B) for nucleophilic groups; in the selective cleavage the negative end of the carbonyl dipole (C) or the imidazole tautomer of the amide acts as an intramolecular nucleophilic agent,
attacking a δ or a γ-carbon (D) atom from which electrons or leaving groups can be displaced (E). (Scheme - 1)

\[ \text{OH} \text{C}^- \text{NH}_2 \rightleftharpoons \text{OH}^- \quad \text{hydrolysis} \]

\[ \delta^- \text{C}^- \text{NH}_2 \rightleftharpoons \text{C}^- \text{NH}^- \]

\[ \text{COOH} + \text{NH}_2^- \]

\[ \text{displacement} \]

\[ \text{Scheme - 1} \]

The side chains can function as sulphur, oxygen and nitrogen nucleophiles in addition and displacement reactions involving various reagents. Basically most of the functional groups are characterised by the same type of reactivity: they are potential nucleophiles or proton
acceptors/donors. This similarity renders it quite difficult for the development of group-specific reagent. An amino, hydroxy or thiol group in a protein always reacts as an electron donor, and the carbonyl function acts as an electron acceptor. Some side chains such as cysteine, methionine, histidine, tryptophan and tyrosine are easily susceptible to oxidation.

Cyanogen bromide (CNBr) as reagent for selective chemical cleavage at methionine residue has gained wide acceptance. Cyanogen bromide is probably the most widely used and most successful chemical cleavage agent. When employed at acid pH, it is extremely selective, reacting exclusively with methionine residues, if sulphydryl functions have been alkylated or otherwise blocked.

The cleavage is quite specific, occurring on the carboxyl side of methionine in yields of 80-100%. However, the reaction is performed in acidic media which are usually denaturing solvents for proteins and peptides. Originally, 0.1 N HCl was employed and more recently, 70% aqueous formic acid or aqueous trifluoroacetic acid are being used as solvents for cleavage by CNBr.
The cleavage reaction undergoes the following course:

\[
\begin{align*}
R\text{NH} & - \text{C} - \text{C} - \text{NH} - \text{CHR'COOH} \\
\text{H}_2\text{C} & - \text{CH}_2 \\
\text{:S} & - \text{C} \equiv \text{N} \\
\text{CH}_3 & /\text{Br} \\
\end{align*}
\]

\[
\begin{align*}
\text{R\text{NH}} & - \text{C} - \text{C} - \text{NH} - \text{CHR'COOH} \\
\text{H}_2\text{C} & - \text{CH}_2 \\
\text{:S} & - \text{C} \equiv \text{N} \\
\text{CH}_3 & /\text{Br} \\
\end{align*}
\]

\[
\begin{align*}
\text{R\text{NH}} - \text{H} - \text{C} - \text{C} - \text{H}_2\text{O} \\
\text{H}_2\text{C} & - \text{CH}_2 \\
\end{align*}
\]

\[
\begin{align*}
\text{R\text{NH}} & - \text{H} - \text{C} - \text{C} - \text{O} \\
\text{H}_2\text{C} & - \text{CH}_2 + \text{H}_2\text{N} - \text{CHR'COOH}
\end{align*}
\]
Modification and cleavage at cysteine residues have been brought about, recently, by 2-methyl-N'-benzene sulphonyl-N'-bromoacetyl quinone diimide under mild conditions with good yield.\(^9\)

The use of N-bromosuccinimide (NBS) in acidic media for the cleavage of tryptophyl, tyrosyl and histidyl peptides has been reviewed.\(^10\)

In proteins, histidyl bonds cannot be cleaved without first cleaving tryptophyl and tyrosyl bonds.\(^10\) When tyrosyl cleavage is prevented by o-dinitrophenylation,\(^11\) o-carbobenzyloxylation or acetylation,\(^12\) NBS at room temperature cleaves only tryptophyl bonds and oxidizes the imidazole ring of histidine residues which on brief heating are cleaved.

The cleavage of tryptophyl peptide bonds with NBS can be depicted in the following manner: (Scheme-3)
Scheme 3
Recently two mild brominating reagents have been developed for tryptophyl peptide bond cleavages in proteins and peptides, without affecting tyrosine or histidine side chains. One of them is the product of N-bromosuccinimide oxidation of 2-(2-nitrophenyl sulphenyl)-3-methyl indole (NPS-skatole). The other mild brominating agent is tribromocresol (2,4,6-tribromo-4-methyl cyclohexadienone; TBC) developed by Burstyn et al.

\[
\begin{align*}
\text{TBC} & \quad \text{BNPS-Skatole}
\end{align*}
\]

Even though these reagents are selective, they suffer from the disadvantage of low yields of cleavage and, when the protein contains several tryptophyl residues, exceedingly complex mixtures are obtained.

NBS in 8 M urea, excess of periodic acid, iodine monochloride, chloramine-T and potassium iodide and iodine and iodate, under suitable conditions cleaved tryptophyl peptide bonds, but again, tyrosine, histidine,
cysteine, cystine and methionine residues were modified and cleaved as well.

A recent addition to this class of cleavage reagents is N-chlorosuccinimide (NCS) and the studies indicate that NCS is the most selective without affecting the tyrosyl or histidyl peptide bonds but with a lower yield of cleavage. 18

The use of NBS for the selective cleavage of tyrosyl peptide bonds, in the absence of tryptophyl and sulphur containing amino acid residues in proteins has been described. Synthetic valyl hypertensin containing one tyrosine residue and no tryptophan or histidine is quantitatively cleaved by NBS. 19 The reaction of NBS with tryptophyl residues can be prevented by reaction of protein with 2-hydroxy-5-nitrobenzyl bromide 20 or by ozonolysis of tryptophyl residues to N-formylkynurenine. 21 This procedure leaves only tyrosyl peptide bonds susceptible to NBS cleavage at room temperature. The cleavage of tyrosyl peptide bonds with NBS in a mildly acidic aqueous medium probably proceeds by the mechanism (1) $\rightarrow$ (6) (Scheme 4). Tyrosine peptides are rapidly brominated with
two equivalents of \( \text{NBS} \) to \( o, o' \)-dibromotyrosyl residues

\[(1) \rightarrow (2)\]. A third equivalent leads to rupture of the

tyrosyl carboxamido bond via intramolecular participation

of the amide carbonyl group on a hypobromite \( (4) \) or tri-
bromodienone \( (3) \) intermediate. An easily hydrolysed

imino lactone \( (5) \) results, which produces the dibromodienone

spirolactone \( (6), R' = \text{NHCO}^- \) and a new \( N \)-terminal

\( (H_3NR) \)

\[ R' = H, \text{NHAc}, \text{NHCOC}_6H_5, \text{NHCO}_2CH_2CH_2CH_2H_5, \text{NHCO}^- \]

Scheme - 4
However, NBS is known to induce other secondary reactions leading to the formation of new crosslinks.\textsuperscript{22} N-iodosuccinimide has been shown to be effective in the cleavage of tyrosyl model peptides.\textsuperscript{23}

Electrolytic oxidation at platinum electrodes also brings about selective oxidative fission of tyrosyl peptide bonds.\textsuperscript{24}

Even though, several reagents have been tried for selective fission of peptide bonds, many of them suffer from lack of specificity and are complicated by side reactions. Hence investigations into the use of new chemical reagents for selective cleavage of peptide bonds are highly desirable.

Careful consideration of the mechanism of known cleavage reactions shows the following common feature: \textit{(Scheme 5)}
An efficient leaving group is present at the $\gamma$ or $\delta$-position to a peptide grouping (7) leading to the weakening of the C-N linkage on removal of the leaving group (8). Hydrolysis of (8) gives a corresponding lactone (9) and a N-terminal amino acid (10). This common mechanism operates in the cleavage reactions initiated by CNBr, NBS, electrolytic oxidations, etc. This mechanistic feature could be exploited in the design of new chemical reagents.
Silver Carbonate on Celite

Silver carbonate on Celite (SCC) was first introduced by Fetizon et al. as an excellent reagent for the oxidation of alcohols. Later, Fetizon and Golfier found that SCC can bring about oxidative coupling of phenols. For example, 2,4,6-trimethyl phenol (11) is oxidised to stilbenequinone (12) in 93% yield (Scheme 6). The intermediate was assumed to be the phenoxy radical (14) as shown by the interception of the radical by oxygen in the oxidation of 2,4,6-t-tributyl phenol (13) to yield peroxide (15) as shown in the Scheme 7.

\[ \text{Scheme 6} \]
It can be expected that SCC can react with tyrosyl residue to generate the phenoxy radical by one electron transfer. This can initiate a fragmentation of adjacent peptide linkage.

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) is a high potential quinone with an oxidation potential of about 1.0 e.v. DDQ is a powerful oxidising agent for phenols. The reaction proceeds smoothly in methanol.
solution at room temperature and usually C-C or C-O coupling are observed. For instance, 2,6-dimethoxyphenol (16) gives diphenone quinone (17) in 62% yield, whereas 2,4,6-t-tributyl phenol (18) gives C-O dimeric product (19) as shown in the Scheme 8.  

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\begin{align*}
\text{H}_3\text{CO} & \quad \text{DDQ} \quad \text{CH}_3\text{OH} \\
\text{16} & \quad \text{H}_3\text{CO} \quad \text{OCH}_3 \\
& \quad \text{17}
\end{align*}
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\begin{align*}
\text{(CH}_3\text{)}_3\text{C} & \quad \text{DDQ} \\
\text{18} & \quad \text{(CH}_3\text{)}_3\text{C} \quad \text{Cl} \\
& \quad \text{(CH}_3\text{)}_3\text{C} \quad \text{OH} \\
& \quad \text{NC} \quad \text{CN} \\
& \quad \text{19}
\end{align*}
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Scheme - 8

DDQ can be expected to cleave tyrosyl peptide bonds, by the intermediate formation of phenoxy radical or by a two stage ionic process.
In the present study, application of these two reagents as potential cleavage reagents for tyrosyl peptide bonds is explored.
1.2 EXPERIMENTAL
4.2.1 Materials

Celite was obtained from Bayer, Germany. Tyrosyl-glycine and Tyrosylalanine were obtained from Sigma Chemicals Co., U.S.A. Crystalline Insulin containing Zn was from Boots, Bombay. DDQ was obtained from Fluka. All the reagents used were of 'AnalaR' grade. Acrylamide and bis-acrylamide were obtained from Fluka. Sephadex G-75 and G-200 were obtained from Pharmacia Fine Chemicals.

UV and visible spectrum were recorded in Pye Unicam SP 1800. IR spectra were recorded in Perkin Elmer 331 grating IR Spectrophotometer. Atomic absorption value was recorded in Varian Techtron AA-6D.

4.2.2 Methods

Preparation of Silver Carbonate in Celite

Celite was purified by washing successively with methanol, containing 10% hydrochloric acid and then with distilled water, until neutral and finally dried at 120°.
Purified Celite (30 g) was added to a stirred solution of silver nitrate (34 g) in distilled water (200 ml). A solution of sodium carbonate (30 g, 105 mmole) in distilled water (300 ml) was then slowly added. The stirring was continued for another 10 minutes. The greenish yellow precipitate was filtered, washed with distilled water until neutral and dried at 60° in vacuum. The reagent contains $\text{Ag}_2\text{CO}_3$ 1 mM/ per 0.57 g of SCC reagent.

Synthesis of Phloretlylglycine

Phloretic Acid

p-Hydroxy benzaldehyde (30 g) and malonic acid (25 g) were dissolved in dry benzene (200 ml). Pyridine (10 ml) and aniline (3 ml) were added. The reaction mixture was refluxed using a Dean and Stark water separator. After 4 hours of reflux, the reaction mixture was evaporated and the residue recrystallised twice from water when p-hydroxy cinnamic acid was obtained, as pale yellow needles. m.p. 197-200°. Yield 75 g.
p-Hydroxy cinnamic acid (7 g) was dissolved in 95% ethanol (40 ml). Pd/c (10%) catalyst (100 mg) was added and the solution was shaken in a Parr hydrogenator, with hydrogen for 3 hours at 45 p.s.i. The reaction mixture was filtered and the solution was evaporated. The residue was recrystallised from aqueous ethanol. The yield of the colourless crystals was 6.2 g. NMR spectrum in D$_2$O showed the disappearance of olefinic protons indicating completion of the hydrogenation.

Phloretic acid was also prepared starting from anisaldehyde.$^{30}$

Anisaldehyde (10.6 g) and sodium (3 g) and ethyl acetate (46 ml) were allowed to react according to the standard method,$^{31}$ except that the reaction mixture was kept below 0° before acidifying. The product was fractionated twice through a wider column, yield 13.2 g (82%). Recrystallised from methanol m.p. 48-50°. p-Methoxy cinnamic ester (10.3 g) in 20 ml of ethanol was hydrogenated quantitatively to ethyl p-methoxyphenylpropionate over Raney Ni within 5 minutes at 80-90° under 100 atmosphere of hydrogen. B.P. 103° (0.1 mm).
Ethyl p-methoxyphenylpropionate (7.8 g) was placed with 34.2 g of HI in a 500 ml flask under an Allihn condenser. The top of the latter was connected to a condenser set for downward distillation. The reaction mixture was heated gently to drive off the alkyl halide formed in the reaction and finally boiled. The mixture of iodides distilling weighed 10.3 g. The desired acid separated out of the cold reaction mixture and was filtered off. After washing with cold water, crystalline solid separated. Yield 6 g. m.p. 122°. Recrystallised from water.

Glycine Methyl Ester Hydrochloride

Glycine (20 g) was suspended in dry methanol (100 ml) and hydrogen chloride gas was passed till the solution was saturated. Most of the solid went into solution and the mixture assumed a pink colour. The solution was then refluxed for 4 hours and the methanol was removed on a water bath. Isopropyl alcohol and methanol were then added in lots and the crystalline solid, which separated was removed by filtration and washed with a little isopropanol.
Phloretylglycine

To a cooled suspension of 1.05 g (0.008 M) of glycine methyl ester hydrochloride in methylene chloride (60 ml), triethylamine (1.2 ml) was added. The mixture was shaken vigorously and then poured into a suspension of phloretic acid (1.33 g, 0.008 M) in 60 ml of methylene chloride. After brief stirring, a solution of \(N,N'-\text{dicyclohexyl} \text{carbodiimide (1.68 g; 0.0084 M)}\) in a small volume of methylene chloride was added. The reaction mixture was stirred at room temperature for 19 hours and then chilled for 5 hours. Following the removal of \(N,N'-\text{dicyclohexylurea}\) by filtration, the filtrate was washed successively with 1 N HCl, 5% sodium bicarbonate solution and water. The solvent was removed under reduced pressure and the mixture was filtered and acidified to pH 7.8 with HCl and extracted with ether. The aqueous layer was then acidified to pH 1 and cooled overnight in refrigerator. The solid that separated was filtered and recrystallised from hot water-methanol mixture. Colourless crystals m.p. 172-174°; yield 0.82 g.

IR, \(\text{Fig. 1} \); NMR, \(\text{Fig. 2} \).
an aqueous solution of phloroethylglycine (10 \mu M) in 1 ml of 20\% acetonitrile/acetate buffer pH 4.8 was shaken with 100 \mu l of SCC reagent on a revolving/shaking 16, 24 and 320 rpm. The sample tubes were collected after 16, 24 and 72 h and the solution was added 1 ml of 0.1 M NaCl solution.

After the absorption was measured for each tube, the samples were spotted against Whatman No. 1 filter paper along with few standard amino acids.
Reaction of SCC Reagent with a Mixture of Amino Acids

Beckman standard amino acid mixture (0.5 μM/ml) was stirred with 100 μM of SCC reagent for 48 hours. The solution was filtered and the amino acid content of the mixture was then determined on the amino acid analyzer. The percentage loss of amino acids were calculated from the values obtained for the standard amino acid mixture and the corresponding values for the SCC treated amino acid mixture. Tyrosine and tryptophan were treated with SCC under varying pH conditions as well as at different temperatures.

Action of SCC Reagent on Phloretylglycine

An aqueous solution of phloretylglycine (10 μM) in 1 ml of 20% acetonitrile/acetate buffer pH 4.8 was shaken with 100 μM of SCC reagent on a revolving shaker (16 rev/min). The sample tubes were moved after 16, 24 and 48 hours. To each tube was added 1 ml of 1 M NaCN solution. UV absorption was measured for each sample. Percentage decrease in absorption at 276 nm was plotted against time. The reaction mixture was then chromatographed on a Whatman No.1 filter paper along with few standard amino acids.
Phloretylglycine also was spotted as control. The solvent
system employed was n-butanol: acetic acid: water (4:1:5).
The paper after drying was sprayed with ninhydrin. The reac-
tion product showed a clear spot for glycine which was absent
in control sample.

Quantitative Estimation of Release of Glycine

The solution (1 ml) after stirring for the
required time interval was layered on a Sephadex G-200
column (11 x 1 cm) and eluted with 0.05 M cyanide-acetate
buffer pH 4.8.

The first 6 ml of the eluate was discarded and
the next 11 ml was collected and concentrated in vacuum.
Glycine content was determined in this fraction by ninhydrin
assay. The column was washed and equilibrated with the
same cyanide/acetate buffer and reused. A control experi-
ment was performed without SCC reagent under identical
conditions and no glycine was detected.
Preparation of Cyanide Acetate Buffer

(1) Stock NaCN - 0.01 M (490 mg/L); (2) Stock acetate buffer - 2700 g of crystalline sodium acetate \((\text{CH}_3\text{COONa \ 3H}_2\text{O})\) was dissolved in two liters of water and 500 ml of glacial acetic acid was added to adjust the pH to 4.8 and was made up to 7.5 litres with water; (3) Cyanide-acetate buffer - 20 ml of the solution (1) was made up to one litre with solution (2).

Ninhydrin Assay of Glycine in the Column Effluent

The following reagents were prepared as follows:

(a) Citrate Buffer: The buffer (0.2 M) was prepared by dissolving 21.0 g of citric acid in 200 ml of 1 M sodium hydroxide and diluting to 500 ml with distilled water. The pH of the solution was found to be 5.0 at 25°C.

(b) Methyl Cellosolve: Technical methyl cellosolve contains ninhydrin positive material and explosive peroxides and these were removed by distillation over
acidulated ferrous sulphate. 30 g of ferrous sulphate crystals were dissolved in 55 ml of water and mixed with 3 ml of concentrated sulphuric acid. 10 ml of solution was added to each litre of the solvent and distilled. The first 5% and the last 10% of the distillate were rejected.

(c) **Preparation of the Ninhydrin Reagent:** The ninhydrin reagent was prepared as described by Moore and Stein by dissolving 0.08 g of Analar stannous chloride in 50 ml of the citrate buffer. This solution was mixed thoroughly with 50 ml of methyl cellosolve containing 2 g of ninhydrin. Then nitrogen/bubbled through the solution and stored in the dark.

(d) **Diluent Solution:** This was prepared by mixing equal volumes of water and alcohol or equal volumes of water and redistilled n-propanol.

(e) **Estimation:** To test solutions, 2 ml of the ninhydrin reagent was added and the solutions were thoroughly mixed by gently shaking the tubes. The tubes were capped and placed in a bath of vigorously boiling water for 20 minutes. The solution was cooled and 5 ml of the diluent solution was added and the whole mixed well.
The optical density of the test solutions were measured at 530 nm. Control experiments were done with standard glycine under identical conditions and from the standard graph the concentration of the glycine in test solutions was calculated.

The treated phloretylglycine (20 ml) was extracted with ether (3 x 25 ml) and the ether was evaporated in vacuum. The residue was taken in methanol and spotted on silica gel G plates (20 x 20 cm) and developed in benzene : methanol : acetic acid (45 : 8 : 4) solvent system. A polar and nonpolar spot were seen on visualization with iodine. The nonpolar fraction was extracted with methanol. A solid material was obtained when the methanol solution was dried in vacuum. IR spectra of this compound was obtained.

### 4.4.4.5 Action of SCC on Dipeptides

Solutions of tyrosylglycine and tyrosylalanine (1 µM/ml) were prepared in water. The peptide solutions (3 ml) were heated with SCC (30 µM) over a boiling water bath under nitrogen atmosphere for 5 hours.
Sodium cyanide solution (0.5 ml of 1 M) was added. The reaction mixture was shaken for an hour and then filtered, through a sintered glass funnel. UV absorption of the treated as well as the control peptide solutions was taken.

The treated peptide solutions were concentrated in vacuum and applied on silica gel-H plates (20 x 20 cm) along with the control peptide solutions and standard glycine and alanine solutions. The plates were developed in the solvent system of butanol: acetic acid: water (80:20:20). The visualisation was done by spraying ninhydrin reagent (0.5% solution in absolute alcohol). The plates were then heated in an air oven at 65° for 15 minutes. The coloured band was scraped out and transferred to test tubes. 6 ml of 75% alcohol containing 0.005% copper sulphate, was added to each tube and the test tubes were shaken mechanically for 15 minutes. The absorbance of the solutions were measured at 550 nm. Estimations were done for standard glycine and alanine solutions under identical conditions and a standard curve was drawn. From the standard curve, the amount of amino acid present in the test solutions was calculated.
Action of SCC on Insulin

Insulin solution (0.25%) was prepared using 0.01 M methanolic hydrochloric acid. 5 ml of the insulin solution was treated with 300 mg of SCC, by shaking at room temperature for 48 hours. After the reaction, sodium cyanide (1 ml, 1 M) solution was added and shaken for one hour. The solution was then filtered through sintered glass funnel. The UV absorption of the filtrate and of the control insulin was obtained.

The end-group assay of the control and treated insulin was done by dinitrophenylating the sample according to Sanger. The pH of the solution was brought to 8.5 by the addition of solid bicarbonate. Dinitrofluorobenzene (0.2 ml) was added to the solution and stirred in the dark for 2 hours. The solution was then extracted three times with ether and the aqueous phase was acidified with conc. HCl to precipitate the dinitrophenyl derivative. The precipitate was centrifuged and washed with water, acetone and ether respectively and dried in a desiccator over P2O5. The precipitate was then hydrolysed with a mixture of 6 N HCl and glacial acetic acid (3 : 1) at 105-110° in
sealed tubes for 16 hours. The hydrolysate was diluted to bring the normality to one and extracted three times with equal volumes of ether. The ether phase was dried in vacuum and the residue was taken in acetone. The aqueous phase was concentrated in vacuum to drive off the acid, and the residue was taken in 0.1 M acetic acid. The organic phase was spotted on silica gel G plates (20 x 20 cm) along with standard DNP-glycine, DNP-phenylalanine DNP-threonine, DNP-leucine and DNP-glutamic acid and developed in the solvent system of chloroform : methanol : acetic acid (95 : 5 : 1). The aqueous phase was spotted on the silica gel plates (20 x 20 cm) along with standard ε-DNP-lysine and o-DNP-tyrosine. The plate was then developed in the solvent system of n-butanol : ammonia (80 : 20). The yellow spots appeared were marked and then sprayed with 0.5% ninhydrin reagent.

**Gel Filtration**

The treated insulin, after the addition of sodium cyanide (1 M, 1 ml), was concentrated in vacuum and dissolved in 1 M formic acid containing 0.1 M NaCl and
applied on a Sephadex G-75 column (height 55 cm; diameter 2 cm void volume 35 ml) and eluted with the same buffer. Fractions (2.3 ml) were collected using fraction collector and the absorbance of the fractions was measured in UV at 276 nm. The experiment was repeated with control insulin. The elution pattern was drawn.

Polyacrylamide gel Electrophoresis

The polyacrylamide gel electrophoresis of the treated and control insulin was done according to the method of Swank and Munkerès. The sample for gel electrophoresis was made by adding 8 M urea, 1% SDS and a drop of β-mercaptoethanol and making up in 0.01 M H₃PO₄ of pH 6.8. The solutions were warmed to 60° for 10 minutes and stored overnight at room temperature.

(a) Preparation of the Reagents

Acrylamide C: Acrylamide (37.6 g) and methylene bis acrylamide (3.76 g) were dissolved in 100 ml water.
Buffer B: A mixture of TEMED (0.6 ml), SDS (0.8 g) and 0.8 M $\text{H}_3\text{PO}_4$ solution was adjusted to pH 5 with Tris and made upto 100 ml.

Reservoir Buffer: $\text{H}_3\text{PO}_4$ solution (0.1 M) was adjusted to pH 6.8 with Tris base and SDS (1 g) was added to one litre of this buffer.

(b) Preparation of the Gel for Electrophoresis

Acrylamide C (16.6 ml) was mixed with buffer B (6.25 ml) and urea (24.2 g). Ammonium persulphate and enough Tris base were added to bring the pH to 6.8 and made upto 50 ml, just before polymerisation.

(c) Apparatus

A disc gel electrophoresis apparatus similar to that described by Davis$^{39}$ was used. The gel tubes were 9 cm in length and 0.5 cm internal diameter and fitted well into the grommets of the buffer reservoir of gel electrophoresis apparatus. The solution was poured into the tubes, and water was layered on the solution to keep it
out of contact with oxygen and to give a flat surface. The solutions polymerised within 30 minutes. The tubes were placed in the electrophoresis apparatus. The treated samples along with the control insulin were layered on the tubes. The tanks of the apparatus were filled with the buffer and the electrophoresis was started, by applying current of 2 mA/gel and run for 5 hours.

After the electrophoresis was completed, as shown by the movement of the dye bromophenol blue added to the samples, the gels were taken out with the help of a syringe or needle by immersing the tube in water. The gels were stained with coomassie blue for an hour and destained with a solution containing 14% acetic acid and 7% methanol.

II.1.1.3 Treatment of Tropocollagen with SCC Reagent

Preparation of Tropocollagen

Rat tail tendons were cut into small pieces and washed with sodium acetate solution (0.5 M) by stirring at 4° for 24 hours. The pieces were then washed with
distilled water by stirring for 24 hours. The tendon pieces were then extracted with acetic acid (0.5 M) by stirring at 4° for 24 hours. The extract was filtered through several layers of cheese cloth and purified as described below.

Collagen was precipitated from the extract by the addition of acetic acid containing 30% sodium chloride. The precipitate was collected by centrifuging at 30,000 r.p.m. for 30 minutes and was again dissolved in acetic acid (0.5 M) by stirring at 4° for 24 hours. The solution was then centrifuged at 30,000 r.p.m. for 30 minutes to remove the impurities. The supernatant was then dialysed against sodium diphosphate (0.02 M) solution with several changes to precipitate the pure collagen. Dissolution in acetic acid (0.5 M) and precipitation with disodium phosphate (0.02 M) were repeated three times. Finally, the solution in 0.1 M acetic acid was lyophilised.
Action of SCC on Tropocollagen

SCC reagent (300 mg) was added to 20 ml of tropocollagen solution (0.3%) and shaken on a rotary shaker at 20° for about 48 hours. Sodium cyanide (1 M, 5 ml) was added to the reaction mixture and again shaken for an hour and then filtered through sintered glass funnel. The filtrate was dialysed against acetic acid (0.05 M) with several changes. An aliquot of the treated collagen was hydrolysed with 6N HCl at 105-110° for 24 hours in sealed tubes under nitrogen. Tyrosine content was determined by the method of Ottaway as well as by alkaline hydrolysis in 0.1 N alkali.

Tyrosine Estimation by Ottaway's Method

The procedure described by Ottaway using 0.3% α-nitroso-β-naphthol solution in acetone and a stabilizing reagent was followed. Stabilizing reagent was prepared by adding 75 ml of acetone to 50 ml of 1 M NaCl and making up the mixture to 300 ml. FeCl₃ (5 ml, 0.5 M) was added immediately before use.
To a solution (2 ml) containing 10–200 µg tyrosine, \( \alpha \)-nitroso-\( \beta \)-naphthol (0.1 ml) was added. The solutions were mixed and placed in a water bath at 60° for 5 minutes. The solution was then removed from the bath and 4 drops of conc. \( \text{HNO}_3 \) were added from a dropping pipette. The solution was returned to water bath for 1 minute. It was then allowed to cool in air for about 5 minutes, after which the stabilizing agent (6 ml) was added. The colour intensity was read after 15 minutes using No.54 green filter in Klett-Summerson photometer. A reagent blank and suitable working standard solutions were run at the same time. The amount of tyrosine present in collagen was obtained from the standard curve.

Alkaline Hydrolysis

A suitable aliquot of the control and treated collagen were taken and 1 N \( \text{NaOH} \) solution was added and diluted to make it 0.1 N \( \text{NaOH} \). The solutions were heated in water bath between 80 and 90° for 15 minutes. After cooling the solutions the UV spectra was taken, recording
between 280 and 300 nm against 0.1 N NaOH. The wavelength of the maximum absorption and the optical density were noted. The tyrosine content was calculated spectrophotometrically according to Bencze and Schmid.\textsuperscript{42}

Polyacrylamide Gel Electrophoresis\textsuperscript{43}

The polyacrylamide gel electrophoresis of the control and treated collagen was done according to the method of Furthmayr and Timpl.\textsuperscript{43} The following reagents were prepared.

**Solution A:** A mixture containing acrylamide (10 g) methylene bis acrylamide (260 mg) and SDS (200 mg) was dissolved in 50 ml of 0.2 M phosphate buffer pH 7.2. TEMED (0.1 ml) was then added and made upto 100 ml in water.

**Solution B:** Ammonium persulphate (150 mg) was added to 50 ml of phosphate buffer (0.2 M) pH 7.2 and made upto 100 ml with water.
Running Buffer: Phosphate buffer (0.1 M, pH 7.2) was prepared and SDS was dissolved in it up to 0.1%. Solution A was heated in warm water and degassed immediately before use. For polymerisation, A and B solutions were added in 1:1 proportion. Samples for electrophoresis were made by adding 0.01 M phosphate buffer pH 7.2, 0.1% SDS, 2 M urea, β-mercaptoethanol (0.5 ml) and heated to 50-60° in a water bath for 15 minutes. A drop of 1% bromophenol blue and few crystals of sucrose were added to the samples. The polymerisation of the gel in the tubes were done as described before (1.2.2.6(c)) and electrophoresis was conducted for 5 hours by applying a current of 6 mA per gel. Removal of the gel, staining and destaining were done, as explained before (1.2.2.6(c)).

Action of DDQ on Phloretylglycine

Phloretylglycine (110 mg, 0.5 mM) was dissolved in 10 ml of 20% aqueous methanol and DDQ (227 mg, 1 mM) was added. The resulting suspension which rapidly turned deep red was stirred magnetically for 5 hours.
The solution was filtered, evaporated to dryness and glycine was identified in it by TLC in the solvent system of butanol : acetic acid : water (80 : 20 : 20).

Experiments with peptides and proteins were complicated by the presence of intense coloured products derived from DDQ whose separation was difficult. These materials interfered with the estimations.
1.3 RESULTS AND DISCUSSION
As part of the programme to study the effect of silver carbonate on Celite (SCC) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) for the possible cleavage of tyrosyl peptide bond, these reagents were tested initially on a dipeptide model phloretethylglycine (23)

1.3.1 Action of SCC on Phloretethylglycine

Phloretethylglycine (23) was prepared by the following route as depicted in Scheme 9.

\[
\begin{align*}
\text{HO-} & \text{CHO} + \text{CH}_2\text{-COOH} \\
\text{Pyridine} & \text{Amine} \\
\text{COOH} & \text{COOH} \\
20a & \\
\text{HCO-} & \text{CHO} + \text{CH}_3\text{COOC}_2\text{H}_5 \\
\text{Na} & \\
21a & R_1 = R_2 = \text{H} \\
21b & R_1 = \text{CH}_3; R_2 = \text{C}_2\text{H}_5 \\
\text{H}_2/\text{catalyst} & \\
22a & R_1 = R_2 = \text{H} \\
22b & R_1 = \text{CH}_3; R_2 = \text{C}_2\text{H}_5 \\
\text{ClH}_3\text{N-CH}_2\text{-COOCH}_3 & \\
1\text{.DCC} & \\
2\text{.OH} & \\
23 & \\
\end{align*}
\]
p-Hydroxybenzaldehyde (20a) was reacted with malonic acid under the catalytic influence of pyridine and aniline. The resulting p-hydroxy cinnamic acid (21a) was hydrogenated using Pd/C as catalyst to give phloreptic acid (22a) which was condensed with glycine methyl ester hydrochloride using the catalyst DCC (N,N'-dicyclohexyl carbodiimide) to give phloretylglycine (23).

Since the reduction of (21a) to (22a) presented difficulties, phloretylglycine (23) was also prepared by another route. Anisaldehyde (20b) was reacted with ethyl acetate in the presence of molecular sodium. The resulting ethyl p-methoxy cinnamate (21b) was hydrogenated using Raney Ni as catalyst, when ethyl p-methoxy/propionate (22b) was obtained. (22b) was demethylated with hydroiodic acid to give phloretic acid (22a) which in the presence of DCC condenses with glycine methyl ester to give phloretylglycine (23).

Exposure of phloretylglycine to the action of SCC reagent resulted in the cleavage of peptide linkage and liberation of glycine as shown by the disappearance of
276 nm absorption (Figure 3) as well as by the increase in the percentage of liberated glycine. The starting phloretylglycine shows an absorption maxima at \( \lambda_{\text{max}} = 276 \text{ nm} \). Its disappearance during the course of the reaction can be correlated with the modification of the aromatic ring due to oxidation. A maximum yield of 80% was obtained after 48 hours of reaction at room temperature (Figure 4).

The oxidative cleavage of phloretylglycine by SCC could be explained by the following mechanism (Scheme 10).

\[
\begin{align*}
\text{23} & \quad \text{NH-CH}_2\text{-COOH} \\
\text{24} & \quad \text{NH-CH}_2\text{-COOH} \\
\text{25} & \quad \text{NH-CH}_2\text{-COOH} \\
\text{26} & \quad \text{NH-CH}_2\text{-COOH} \\
& \quad \text{NH}_2\text{-CH}_2\text{-COOH}
\end{align*}
\]

Scheme 10
FIG-3. CHANGES IN ULTRAVIOLET ABSORPTION OF CLEAVAGE YIELDS OF PHLORETYLGLYCINE TREATED WITH S.C.C.
In agreement with this mechanism which requires the formation of dienonalactone (22), the IR spectrum (Figure 5) of the nonpolar residue isolated in poor yield by preparative thin-layer developed paper, shows the absence of the aldehyde carbonyl absorption present in the starting material at 1630 cm\(^{-1}\) (amide I band). The characteristics of this material as the dienonalactone (22) are supported by the peaks at 1750 cm\(^{-1}\) (lactone ring), 1680 cm\(^{-1}\) (conjugated carboxyl), and 1650 cm\(^{-1}\) (conjugated amide). The poor yield of dienonalactone may be due to its further reaction catalysed by SCC, probably initiated by coordination of silver ions with the carbonyl groups so-called "sickle reaction" of dienonalactone (22) to original planar dicarboxylic compound as noted earlier. The \(R_f\) value of this compound agrees well with the reported value (0.60–0.70) for dienonalactone (22) obtained under the same TLC conditions. Use of dienonalactone could not be seen however in IV.

This is probably due to the fact that the peak on 1228 we refer in the above figure is not the dienonalactone but another compound whose \(R_f\) value is lower. However, in keeping with the formation of dienonalactone, the alkene bond of this compound will be obscurred by halogen acid and acetic acid.

FIG. 4. CORRELATION OF LOSS OF ABSORBANCE AT 280 NM (○→○) WITH THE LIBERATION OF GLYCINE (○→○) IN THE OXIDATION OF PHLORETYLGLYCINE WITH SCC.
In agreement with this mechanism which requires the formation of dienonelactone (27), the IR spectrum (Figure 5) of the nonpolar fraction isolated in poor yield by preparative thin layer chromatography, showed the absence of the amide carbonyl absorption present in the starting material at 1630 cm\(^{-1}\) (amide I band). The characterization of this material as the dienonelactone (27) is supported by the peaks at 1750 cm\(^{-1}\) (lactone carbonyl), 1680 cm\(^{-1}\) (conjugated carbonyl), and 1650 cm\(^{-1}\) (conjugated C=C). The poor yield of dienonelactone may be due to its further reaction catalysed by SCC probably initiated through coordination of silver ions with the carbonyl oxygen. Facile reversion of dienonelactone (23) to original phenolic compound is noted earlier. The \(R_f\) value of this compound agrees well with the reported value (0.66-0.7) for dienonelactone (27) obtained under the same TLC conditions. The characteristic absorption maxima at \(\lambda_{226}\) due to dienonelactone could not be seen however in UV. This is probably due to the fact that the peak at \(\lambda_{226}\) may become submerged in the much more intense absorption in the shorter wavelength. It has already been reported that spectroscopic assay of the dienonelactone will be obscured by halogen acid and acetic acid.
The oxidation brought about by the reagent silver carbonate on Celite, involves the removal of one hydrogen from phenol (24) followed by participation of the amide carbonyl group (26). It can be removed as a free radical or $H^+$ ion. This reaction being heterogeneous in nature, adsorption plays an important role. Addition of Celite might involve increase in the surface area.

It has been noticed earlier that the amount of Celite on which silver carbonate is precipitated is increased, an enhancement in the oxidation is observed until a maximum is reached. Hence, adsorption is important in this oxidation.

Similarly polarity of the solvent should influence the degree of adsorption of reactants and the rate of the desorption of the products. It is very difficult to predict the exact mechanism because of several factors. Silver carbonate can exist in two forms and the proportion of the two form is a function of mode of preparation. It is not possible to achieve uniformity in the size and shape of the adsorbent particles. Thirdly, the rate of agitation will also influence the yield. $Ag^+$ in the solid phase can constitute active sites for the chemisorption
of the phenol oxygen. Increase in temperature can increase the rate of oxidation and desorption of the product and increase in the rate of oxidation. It is to be noted that mere addition of Celite to silver carbonate does not catalyse the reaction. The silver carbonate must be precipitated in the presence of Celite. This method increases the number of active sites on the surface. Other silver salts like acetate, formate, and oxalate, similarly precipitated are totally ineffective in these oxidation reactions.

The cleavage process can be delineated in the following way: (a) a reversible adsorption of substrate on the Celite; (b) oxidation of the substrate by silver ions; (c) desorption of the product from the Celite surface. The excess of the active reagent (silver carbonate) is kept away from the solution due to its immobilization on the Celite. Addition of sodium cyanide probably helps in the desorption of the product after the reaction.
It may be of interest in this connection to compare the action of silver salts with amino acids and peptides. Herbst and Clarke have reported the oxidation of amino acids with silver oxide at elevated temperatures. Amino acids are found to undergo decarboxylation and sometimes deaminations. 49

1.3.2 Action of SCC on Amino Acids

When a solution of the standard amino acid mixture was treated with SCC reagent, the amino acids sensitive to oxidation such as tyrosine, histidine, methionine, tryptophan and cysteine suffered 74, 40, 30, 100 and 100% loss respectively as revealed by the amino acid analysis.

Analysis of the product in the amino acid analyzer showed additional peaks attributable to cysteic acid, methionine sulphoxide and sulphone. The yields of these products could not be calculated due to the nonavailability of the standard samples in the amino acid analysis.
The action of SCC reagent on individual amino acids greatly depends on the pH, temperature and concentration of the reagent. The product obtained under extreme basic conditions showed additional absorption peaks at longer wavelength. Highly acidic conditions cannot be used due to the sensitivity of silver carbonate. At higher temperature the products formed showed also minor absorption in the visible region. Figure 6 and Figure 7 show the UV absorption pattern of the control and treated tyrosine and tryptophan at room temperature and at 80°. The ninhydrin assay of the control and the treated amino acids correlated with the reduction in the UV absorption.

1.3.3 Action of SCC on Dipeptides

When tyrosylglycine in aqueous medium was treated with SCC at room temperature, a negligible reduction in the UV absorption was noticed. Even conducting the reaction on a water bath at 50 to 60° did not improve the yield. The reaction was found to proceed only on a boiling water bath and a loss of about 7-8% was found by
The changes in ultraviolet absorption of tyrosine following SCC treatment.

- TYROSINE (CONTROL)
- TYROSINE TREATED WITH SCC AT 30°
- TYROSINE TREATED WITH SCC AT 80°

FIG - 6
IV. Absorption (Figure 4) and by the ninhydrin assay of the released glycine. Similar observations were made with tyrosylalanine. In the presence of the unreacted dipeptide in the product, compared with the ninhydrin assay of the released N-terminal amino acid. The released glycine and alanine could not be separated from the unreacted dipeptides either by gel filtration or by ion exchange chromatography because of their close similarity in their molecular weight and ion exchange behaviour. The separation could only be achieved through thin-layer chromatography using silica gel without heating.

The yield obtained represents a minimum since some low yield have occurred on preparative thin layer chromatography followed by extraction of amino acid spots and its estimation colorimetrically.

The low yield of cleavage is presumed to be due to the nonprotection of the N-terminal of the tyrosine moiety and subsequent intermediates.

Similar low yield of cleavage have been observed with Watanabe as well as in the electrophilic cleavage of the peptide having the tyrosyl residue. In the present case these results were converted into a 3,7-dinitrotyrosyl derivative.

FIG-7

THE CHANGES IN ULTRA VIOLET ABSORPTION OF TRYPTOPHAN FOLLOWING SCC TREATMENT

- - - TRYPTOPHAN (CONTROL)
O-O TRYPTOPHAN TREATED WITH SCC AT 38°
× × TRYPTOPHAN TREATED WITH SCC AT 80°
UV absorption (Figure 8) and by the ninhydrin assay of the released glycine. Similar observations were made with tyrosylalanine. The presence of the unreacted dipeptide in the product interfered with the ninhydrin assay of the released N-terminal amino acid. The released glycine and alanine could not be separated from the unreacted dipeptides either by gel filtration or by ion exchange chromatography because of their close similarity in their molecular weight and ion exchange behaviour. The separation could only be achieved through thin layer chromatography using silica gel without binder. The 7-8% yield obtained represents a minimum since some loss would have occurred on preparative thin layer chromatography, followed by extraction of amino acid spots and its estimation colorimetrically.

The low yield of cleavage is presumed to be due to the nonprotection of the N-terminal of the tyrosine moiety and subsequent formation of other intermediates. Similar low yield of cleavage had been observed with NBS as well as in the electrolytic oxidation on the dipeptides having the unprotected N-terminal tyrosyl residues. In the former case the N-terminal tyrosyl residues are converted into a 5,7-dibromo-6-hydroxyindole derivative.
Having absorption maximum at 350 nm, this conversion was assumed to proceed by the initial oxidation to a tribromodiglycine intermediate followed by an external Michael-type addition of the amino function.

To determine whether any binding of silver ions takes place under the conditions of the cleavage experiments, the amount of bound silver in treated tyrosylalanine solution was determined by atomic absorption method. The low value (0.57%) obtained suggests minimal uptake of silver ions in present experiments. It may be noted that even in wool which contains many cystine residues, the amount of silver bound is limited to 10% by equilibrating with aqueous silver nitrate solution for 24 hours. 51,52,53,54,55 E isError and Mason have shown that most amount of silver bound resulted in reversible complex formation with carboxyl groups accompanied by H+ release. 52 Thus, though the yields are limited, the stripping about be occurring in the case of dipeptides. In this context, it would be instructive to consider the basic requirement of a chemical reaction in solution of peptide bonds in peptides and proteins.

Such enzymes act preferably in aqueous medium without excessive pH and temperature.

(a) TYROSYLGlycINE
(b) TREATED TYROSYLGlycINE

FIG-8
having absorption maximum at 350 nm. This conversion was assumed to proceed by the initial oxidation to a tribromo-dienone intermediate followed by an internal Michael-type addition of the amino function.

To determine whether any binding of silver ions takes place under the conditions of the cleavage experiments, the amount of bound silver in treated tyrosylalanine solution was determined by atomic absorption method. The low value (1.57%) obtained suggests minimal uptake of silver ions in the present experiments. It may be noted that even in wool which contains many cystine residues, the amount of silver bound is limited to 10% on equilibrating with aqueous silver nitrate solution for 24 hours. Simpson and Mason have shown that major amount of silver bound resulted in reversible complex formation with carboxyl groups accompanied by H⁺ release. Thus, though the yields are limited, the cleavage was brought about by SCC in the case of dipeptides. In this context, it would be instructive to consider the basic requirement of a chemical reagent for selective cleavage of peptide bonds in peptides and proteins. Such a reagent should act preferably in aqueous medium without extremes of pH and temperature
which will denature proteins. Further, the reagent should exhibit specificity of the cleavage. Viewed in this light, SCC shows several advantages such as mildness of action at ambient pHs. Further, cleavage yields are moderate as shown by reaction with phloretylglycine. In this oxidation, since the product derived from the anion portion of the oxidant is carbonic acid which is eliminated as carbon dioxide, acidity of the medium is unaffected by the reaction. Although a 1924 patent reported use of chemical reagents on porous carriers, it is only recently that interest has been rekindled in this area as shown by development of several reagents either immobilized on porous solids like Celite, ion-exchange resins, zeolites or intercalated in graphite to bring about selective, mild and convenient transformations. The function of the carriers is to lower the entropy of activation when the reactant and the reagent are adsorbed close to each other and in the proper orientation for chemical reactions.

In order to define the specificity of the performance of SCC a study of its action was extended to proteins.
1.3.4 Action of SCC on Proteins

(a) Insulin

Insulin is a protein hormone containing few strategically located tyrosyl residues and hence an ideal test case to demonstrate the specificity of this cleavage process Figure 3.

Insulin, dissolved in 0.01 N hydrochloric acid containing 10% methanol, was subjected to the action of SCC. Figure 6 shows the UV absorption patterns of the control and treated insulin. There is a limited reduction in the UV absorption after the treatment, probably brought about by the cleavage of tyrosyl peptide bonds and subsequent oxidation of the tyrosyl moiety.

When the cleaved products were analysed qualitatively by FDNB method, using thin layer chromatography, only glutamic acid was identified as DNP derivative, in addition to the original DNP derivatives of glycine and phenylalanine, in the organic phase. The aqueous phase showed the presence of o-DNP-tyrosine and ε-DNP-lysine. This indicates that the cleavage was incomplete, however,
FIG 9 The amino acid sequence of bovine insulin
The absence of tyrosine residues in insulin is evidenced by the absence of absorbance at 278 nm and leucine is present in this protein, so that a tyrosyl-glutamine residue (Tyr-Glu-Asp) (Figure 7) is accessible to the reagent, thus exhibiting a very high degree of specificity in attacking the surface of the molecule.

It may be noted that reversible adsorption of tyrosyl hydrazide on Celite is a prerequisite for the cleavage. Hence, all the tyrosyl peptide bonds cannot be expected to be accessible for cleavage by the reagent.

Desorption of Celite prevents entry of insoluble Ag⁺ from gaining access to all parts of folded insulin. In the electrolytic oxidation of insulin, tyrosine, tyrosyl-leucine, tyrosyl-leucine, and tyrosyl-cystine were modified but tyrosyl-glycine was left unaltered. The use of SCC and the cleavage of tyrosyl peptide thus complements the cleavage of insulin by electrolytic processes.

**FIG-10**

---

**O** - INSULIN (Control)

**X** - INSULIN TREATED WITH SCC.

AT 30°.
still restricted to tyrosyl peptide bonds. The absence of DNP-cysteine derivative may be due to the complete oxidation of cystine residues in insulin. The absence of threonine and leucine DNP derivatives may mean that sufficient tertiary structure is present in this protein, so that only tyrosyl-glutamine residue (Tyr-Gln-A₁₄) (Figure 9) is accessible to the reagent, thus exhibiting very high degree of selectivity in attacking the surface of the molecule. It may be noted that reversible adsorption of tyrosyl hydroxyl on Celite is a prerequisite for the cleavage. Hence, all the tyrosyl peptide bonds cannot be expected to be accessible for cleavage by the reagent. Immobilization on Celite prevents entry of insoluble Ag⁺ from gaining access to all parts of folded insulin. In the electrolytic oxidation of insulin, tyrosyleucine, tyrosylthreonine and tyrosylcystine were affected but tyrosylglutamine was left unaffected.³⁴ The use of SCC for the cleavage of tyrosyl peptide thus complements the cleavage of insulin by electrolytic procedure.
In this context, the size and the tertiary structure of the protein molecule assume importance. Many of the residues are shielded from solvation (buried) and relatively inactive towards the reagent, dissolved therein. Data had been collected by X-ray crystallographic analysis of eleven proteins and the average exposure of side chain atoms to solvent was calculated, which reflects the degree of 'buriedness' for each type of amino acid residue. The average percentage composition of tyrosine in such a study had been calculated as 3.4 and its fractional exposure which gives the ratio of the exposure in the folded molecule to the exposure in the unfolded model, as 0.25. The latter value is moderate compared to the values given for Lysine (0.58), Glutamine (0.43), Glutamic acid (0.49), Serine (0.43), Phenylalanine (0.17), Methionine (0.11) and Tryptophan (0.11). Although the hydroxyl function of Tyr-Gln (A) (Figure 9) is unreactive, electrophilic reagents preferentially react with the tyrosyl ring of A. Related studies by Gattner, showed that the reactivities at pH 7 to pH 3 as Tyr > Tyr > Tyr > Tyr . Similar selectivity in oxidation might be
displayed by the reagent developed. Additional selectivity might be imposed since the reagent is not in solution and hence further constrained in its approach to various residues in different environments.

SCC treated insulin was subjected to gel filtration using Sephadex G-75 and the elution pattern obtained is shown in Figure 11. The first fraction corresponded closely to the control insulin. The second small fraction could be due to the fragment consisting of 13 amino acid residues after the cleavage at A14 Tyr-Gln. The large fragment might have overlapped with the unreacted insulin.

The polyacrylamide gel electrophoresis of the SCC treated insulin, following the method of Swank and Munkres does not show any new bands other than in the control indicating that not many fragments have resulted from the cleavage of insulin (Figure 12).
Figure 12 - Gel Electrophoresis pattern of
(a) Control insulin
(b) Treated insulin
(b) **Collagen**: Collagen is the major structural protein present in the mammalian body. Collagen molecule consists of essentially a triple helix or coiled coil in which three helical polypeptide chains are coiled into a super helix. The whole rope-like structure is stabilised by interchain hydrogen bonding as well as small number of covalent cross links. The intramolecular cross linkages which occur naturally in collagen are apparently located mainly at the N-terminal ends of the molecules, in short non-helical segments which are termed "the telopeptide region" (Figure 12). Thus, treatment of collagen containing $\beta$ and $\gamma$ components with proteases such as pronase or pepsin results in the decrease in the number of $\beta$ and $\gamma$ components indicating the removal of cross linking regions of the chains. This evidence confirms that the attack occurs only at the ends of the molecule and the digested portion contains the natural cross links in the molecules. The telopeptide region is rich in tyrosine. Thus it is reasonable to expect these relatively tyrosine-rich portions of collagen molecule play an active role in the stabilization of collagen. As the SCC reagent
SEQUENCE OF COLLAGEN TEOLOPEPTIDE REGION

N-TERMINAL

GLU-MET-SER-TYR-GLY-TYR-ASP-GLU-LYS-SER-ALA-GLY-VAL-SER-VAL-PRO

C-TERMINAL

SER-GLY-GLY-TYR-ASP-LEU-SER-PHE-LEU-PRO-GLN-PRO-PRO-

GLN-GLN-GLX-LYS-ALA-HIS-ASP-GLY-GLY-ARG-TYR-TYR

FIG - 13
shows a propensity for the oxidative cleavage of tyrosyl peptide bonds, a study of the reaction of collagen with this reagent may be of interest with regard to the stabilization processes peculiar to collagen. Similarly the antigenic response in infected heterologus tropocollagen is directed against the telopeptide region \(^{66}\) and tyrosine is frequently associated with the antigenicity in protein. \(^{67}\) So, any modification of the tyrosyl residues in the telopeptide region is also of interest from the point of view of antigenic properties of collagen.

SCC treated collagen showed a decrease in tyrosine content, when compared with native collagen (Table I).

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
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<tbody>
<tr>
<td>Tyrosine Content of Native and Treated Collagen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tyrosine content %</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Native tropocollagen</td>
<td>0.5</td>
</tr>
<tr>
<td>(2) SCC-treated tropocollagen</td>
<td>Nil</td>
</tr>
</tbody>
</table>
Polyacrylamide gel electrophoresis of the SCC treated collagen showed no difference in the band pattern compared to the native collagen (Figure 14). This evidence confirms that the attack occurs only at the ends of the molecule and the digested portion contains the natural cross links in the molecule. Here again, high selectivity of this reagent towards tyrosyl residues is indicated.

1.3.5 Action of DDQ on the Model Peptide, Phloretylglycine

When a solution of phloretylglycine in aqueous methanol was treated with DDQ for 5 hours, the solution developed an intense red colour. Glycine was isolated in nearly quantitative yield from this reaction by preparative thin layer chromatography. Insulin was similarly treated with DDQ at neutral pH. Though cleavage occurred, the fragments could not be separated easily by gel filtration because of the presence of intensely coloured quinols whose removal from the peptide fragments presented difficulty. Several attempts were made to get over this problem. Further work is needed to convert the quinols to colourless
Figure 14 - Gel Electrophoresis pattern of
(a) Calf-skin collagen
(b) Treated collagen
non-interfering species probably by reductive methods.

The probable mechanism for cleavage of phloretylglycine similar to by DDQ might involve either one electron transfer/ Scheme 9 or a two-stage ionic process as depicted in the Scheme 11.
1.4 REFERENCES


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1.5 SUMMARY
Two new reagents, silver carbonate on Celite (SCC) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) were tried for the potential cleavage of tyrosyl peptide bonds. Phloretylglycine (23) was cleaved by SCC reagent with liberation of 80% glycine. The probable course of the mechanism was indicated by the isolation of spirodienone lactone (27). The specificity of this cleavage process was established by extending the reaction to dipeptides, insulin and collagen. Insulin was specifically cleaved at Tyr-Gln (A_{14}) linkage, while collagen was affected only at the telopeptide region. The reaction with SCC proceeded under very mild conditions. DDQ cleaved phloretylglycine quantitatively. These two reagents have the potential to be developed as selective cleavage reagents for proteins containing tyrosyl peptide linkage.