techniques, such as the Cooley-Tukey algorithm. Although we do not discuss the details here, the subject of Fourier spectroscopy is as full of pitfalls on the mathematical side as on the experimental (*14*).

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Chemical Cleavage of Proteins

Selective fragmentations and modifications reveal structure.

Bernhard Witkop

Proteins, such as enzymes, hormones, immunoglobulins, or the structural tissue component collagen, are biopolymers composed of about 20 different amino acids. The repeating sequence

$$H_2N\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}CO\text{-}[NH\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}CO]_n\text{-}NH\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}CO_2H$$

is held together by the amide group as the covalent link. The aim of the protein chemist is to establish the primary structure, that is, the correct order of all amino acids in the chains between the amino-terminal and the carboxy-terminal residues. Proteins are digested, that is, hydrolyzed, by specific mammalian enzymes (trypsin, chymotrypsin), by less specific plant and animal enzymes (papain, pepsin, elastase), or by very aggressive bacterial enzymes (pronase, subtilisin) which hydrolyze all peptide bonds indiscriminately. The chemical cleavage of the amide or peptide group requires strong acid or base and elevated temperature.

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By controlling the conditions of acid hydrolysis, Emil Fischer was able to show that, for instance, silk fibroin which is rich in serine, easily breaks down into peptides—aggregates of two, three, or more amino acids which reveal part of the sequence of the original protein (*1*). The extension of this principle, the matching of a sufficient number of overlapping peptides, permits the establishment of the complete primary sequence of a protein. Historically, the sequences of the A and B chains of the protein hormone insulin with a total of 51 amino acids were first elucidated in this fashion (*2*).

Selective nonenzymatic cleavage: cyanogen bromide reaction. A much larger protein is the hormone that regulates and controls growth in humans (Fig. 1) (*3*). It contains 188 amino acid residues. In such a protein, tryptic digestion alone, that is, hydrolysis by trypsin, which breaks the protein at the arginine and lysine residues, would lead to a complicated mixture of peptides and to a major problem of separation. Cyanogen bromide (Br-C≡N), a simple chemical reagent, permits almost quantitative cleavage (*4, 5*) next to the three methionine residues, Met(14), (122), and (167). The four peptide fragments formed in this reaction are separable by column chromatography and are then amenable to individual sequence analysis by enzymatic digestion (*3*). No generally usable enzymatic cleavage is known which would be of comparable selectivity. Such a reagent makes possible and simplifies the arduous task of "sequencing" large proteins with molecular weights as high as 50,000 to 150,000, that is, with about 500 to 1500 individual amino acids (Table 1) (*6*).

Besides aiding in the establishment of the primary sequence, the products of cyanogen bromide cleavage provide answers to other questions, such as whether the activity of human growth hormone resides in the entire structure or whether it is possible to have smaller active fragments (*7*). Adrenocorticotropic hormone (ACTH), which contains 39 amino acids, requires only 23 amino acids for physiological activity (*8*) as established by synthesis.

Rabbit immunoglobulin of type G (IgG) with a molecular weight of 150,000, is cleaved by cyanogen bromide (*9*), as well as by papain and pepsin. From an immunological point of view, this reduction in size has advantages. All its undesirable properties, such as complement fixation, skin binding, and reaction with antiglobulin factors, stay in the fragments which remain on the column on gel filtration (*10*). The eluted smaller immunoglobulin may now be an improved natural therapeutic agent whose practical application is open to clinical testing. Cleavage by cyanogen bromide of 18 of the 20 methionyl peptide bonds in the two heavy (mo-

lecular weight, 50,000 each) and two light chains (molecular weight, 24,000 each) of human IgG has yielded almost the complete sequence (Fig. 2) and has provided a chemical basis for antibody diversity and specificity (*11*).

Conversely, the smaller fragment obtained by selective cleavage of a large protein may be the part of physiological interest. The process of inflammation caused by wounds, foreign intruders, or disease, has been described in terms of general breakdown of large protein molecules. Some of these fragments have direct pain-producing or muscle-contracting activity. The bradykinin and kallidin from human plasma belong to this class of oligopeptides and consist of nine to ten amino acids. Cleavage of plasma by cyanogen bromide releases fragments with kinin activity from the precursor protein, namely, human (*12*) and bovine (*13*) kininogens whose molecular weight is about 50,-

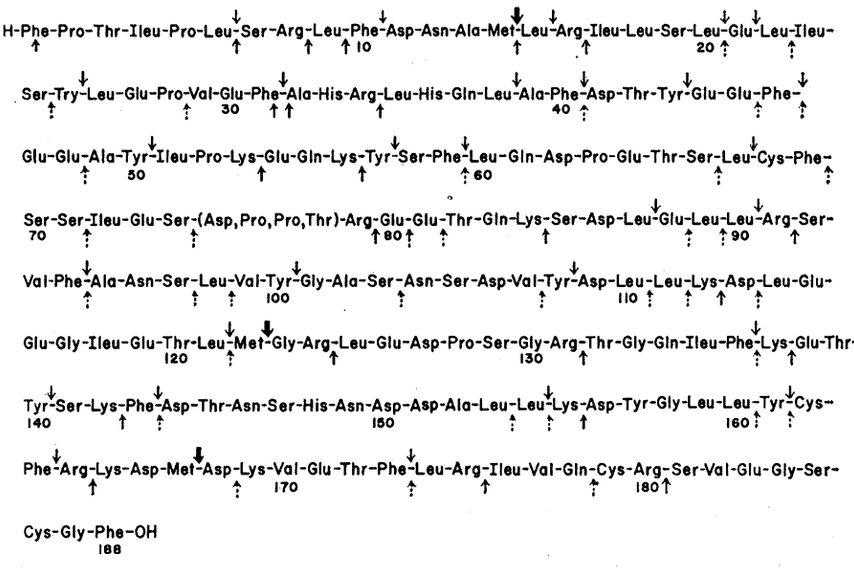


Fig. 1. The primary structure of growth hormone from humans. The (dotted) arrows below the lines indicate enzymatic cleavage by trypsin (and pepsin); the arrows above the lines, attack by chymotrypsin. The boldface arrows show the selective cleavage by cyanogen bromide next to Met(14), Met(122), and Met(167).

Table 1. Cyanogen bromide cleavage of peptides and proteins. R.T., room temperature.

Peptide or protein	Molecular weight	Met residues	Reaction conditions				Reference
			Solvent	BrCN/Met	Temperature (°C)	Time (hr)	
Ribonuclease (bovine pancreatic)	13,700	4	0.1*N* HCl	30	25	24	(*49*)
Active site peptide of rabbit muscle aldolase	3,000	1	0.1*N* HCl		R.T.	24	(*50*)
Rabbit muscle aldolase (4 units at 40,000)	160,000	4 × 3	70% HCOOH	30	R.T.	22	(*50*)
Azurin	14,000	6	0.1*N* HCl		R.T.	24	(*51*)
Carboxypeptides A	34,600	3	70% HF		R.T.	24	(*52*)
Chymotrypsin	25,000	2	0.1*N* HCl	<30	R.T.	24	(*53*)
Collagen	100,000	6–8	0.1*N* HCl	100	30	15	(*54*)
Cytochrome c	12,000	2	0.1*N* HCl		40	24	(*55*)
Baker's yeast cytochrome c	12,000	2	0.1*N* HCl		36	24	(*56*)
Pseudomonas cytochrome c-551	8,000	2	0.1*N* HCl	<30	R.T.	24	(*57*)
β-Galactosidase	135,000	24	70% HCOOH	50	R.T.	16–20	(*58*)
Gastrin	2,000	2	Aqueous HF		R.T.	24	(*59*)
Immunoglobulin IgG (rabbit):							
Partial cleavage, active fragment	~150,000	10	0.3*N* HCl	200	R.T.	4	(*9*)
Complete cleavage, inactive fragment	150,000	10	70% HCOOH		R.T.	24	
Myoglobin	18,000	2	0.1*N* HCl	40	R.T.	24	(*60*)
Parathyroid hormone	9,000	2	0.1*N* HCl		R.T.	24	(*61*)
Cross-linked bovine pancreatic ribonuclease	14,000	4	0.1*N* HCl		R.T.	24	(*62*)
Bovine pancreatic trypsinogen	24,000	2	0.2*N* HCl	40	30	30	(*63*)
Extracellular nuclease (*Staphylococcus aureus*)	16,500	4	70% HCOOH	30	25	20	(*64*)
Porcine thyrocalcitonine	3,600	1	70% HCOOH	180	25	24	(*65*)
Thioredoxin	12,000	1	(CF_3COOH) 70% HCOOH	300	25	24	(*66*)
Cholecystokinin-pancreozymin	3,500	3	0.1*N* HCl		21	24	(*67*)
α-Lactalbumin	16,000	1	60% HCOOH		R.T.		(*68*)
Human kininogen	50,000	2	0.25*N* HCl	5	35	20	(*12*)
Human growth hormone	21,500	3	70% HCOOH		R.T.		(*3*)
Tryptophan synthetase (fragment)	2,000	1	0.1*N* HCl	30	R.T.	24	(*69*)
Calf thymus (GAR) histone	10,600	1					(*70*)
Bovine growth hormone		4					(*71*)
S-Methylmethionine-29 ribonuclease A	13,700	4	0.1*N* HCl	30	R.T.	24	(*72*)
Lysozyme (hen egg)	14,500	2	70% HCOOH				(*73*)
Human immunoglobulin IgG	150,000	20	70% HCOOH		R.T.	4	(*74*)

Table 2. Cleavage of peptide bonds next to cysteine of modified ribonuclease (*18*). Step 4 of the cleavage procedure was performed with *o,o'*-diaminodiphenylamine or with hydrogen peroxide. Numbers in parentheses denote the position of the residue in the peptide chain.

NH_2-terminal amino acid	Yield (mole/mole protein)		
	Dinitrophenylation		Picrylation
	Alkaline H_2O_2	*o,o'*-Diamino-diphenylamine	Alkaline H_2O_2
Lysine (41, 66)	0.95	1.2	0.52
Arginine (85)	0.15	0.28	0.18
Asparagine (27)*	0.34	0.58	0.23
Serine (59)	0.42†	0.78	0.40
Glutamic acid (111)	0.52	0.38	0.19
Alanine (96)	0.24	0.48	0.35
Tyrosine (73)	0.10‡	0.32	0.05‡

* Found as aspartic acid. † Uncorrected. ‡ Found only when protected by phenol or phloretic acid from oxidation step 4.

000. The cleavage that occurs between two methionine residues has been formulated as shown in Fig. 3. The newly released pentadecapeptide has about 10 to 15 percent of the kinin activity that native human plasma shows when it is treated with kallikrein, an enzyme that specifically releases the more active nonapeptide bradykinin (residues 2–10, Fig. 3).

Mechanism of nonenzymatic cleavage: cyanogen bromide reaction. In order to understand the principle of selective chemical cleavage, it is necessary to review the rules of general hydrolysis of amide bonds. The amide group is a dipole with the negative charge residing on oxygen, and with the positive charge localized partly on the carbonyl carbon and partly on the amide nitrogen (Fig. 4). To this dipole can be added the (anionic) hydroxyl (HO^-) of alkali, or the (cationic) proton of an acid. In both alkaline and acidic hydrolyses, the unstable tetrahedral intermediate breaks down into a carboxylic acid and another amino-terminal residue. There is generally no selectivity in the indiscriminate addition of H^+ or HO^- to the many amide dipoles of a protein chain. In the selective chemical cleavage the principle is reversed: the negative end of the dipole, the imidolate anion, seeks for an electrophilic center within the molecule. Such an electrophilic center can be created by a suitable leaving group, $X \rightarrow X^-$. The departure of X and the intramolecular displacement need not be separate discrete steps, but may be concerted. The preferred mechanism is 1,5-interaction which leads to thermodynamically most favored five-membered ring systems, such as an imino-γ-lactone. The group in a methionine peptide that is being removed is the sulfur function (Fig. 5). In order to facilitate the departure of sulfur, the cyano-sulfonium intermediate is formed by interaction with the pseudohalogen, cyanogen bromide. The displacement of the volatile and stable methyl thiocyanate, CH_3-S-C≡N, under the concerted attack of the imidolate anion leads to the unstable imino-γ-lactone which spontaneously hydrolyzes to homoserine (lactone) and a new amino terminal residue.

Selective cleavage of cysteine peptides. This principle can be extended to the cleavage of cysteine peptides (*14*). In this case the sulfur has to be methylated to *S*-methylcysteine, the lower homolog of methionine. On reaction with cyanogen bromide, 1,5-interaction with the amide carbonyl can occur only with the *N*-acyl group (Fig. 6). Instead of the imino-γ-lactone, an oxazolinium intermediate is formed which is easily converted to an *O*-acyl derivative of serine. Acid hydrolysis leads to a carboxy-terminal and an amino-terminal serine peptide. Cleavage occurs at the amino peptide bond of the cysteine residue, in contrast to cleavage at the carboxyl peptide bond of the methionine residue. The cyanosulfonium intermediate, especially at elevated tem-

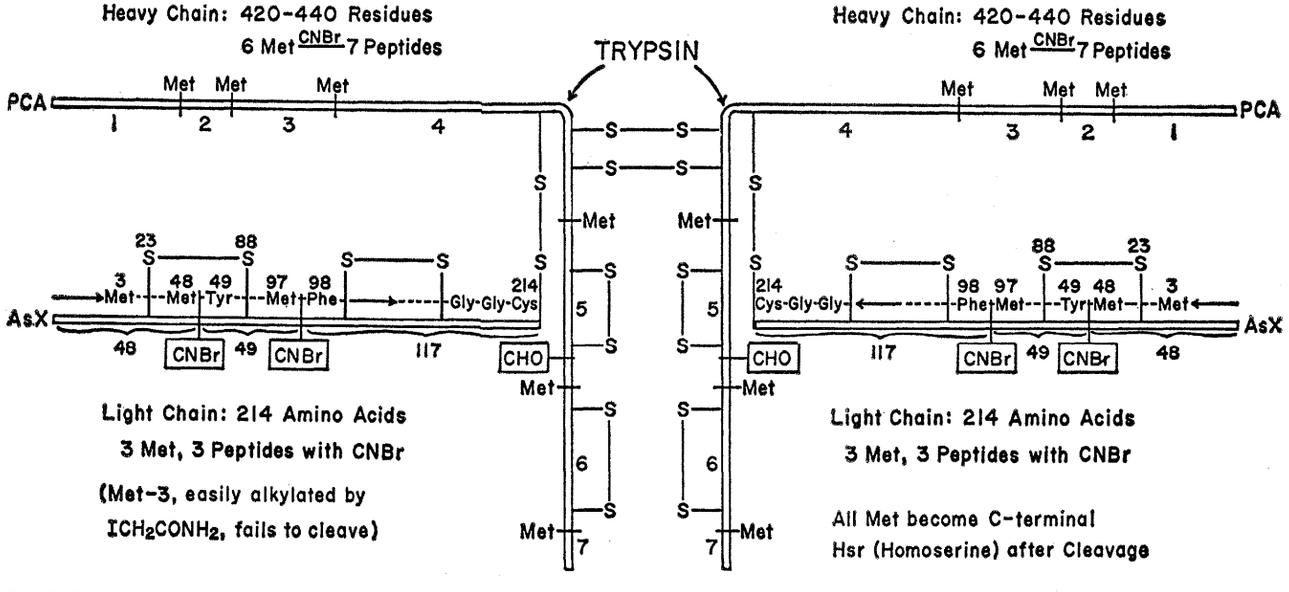


Fig. 2. Linear model of the two light and two heavy chains of the human γG-immunoglobulin Eu. Cyanogen bromide cleavage has made possible the establishment of the major sequences of a total of ~1300 amino acids (*74*). The removal of peptides 5, 6, and 7 by cyanogen bromide cleavage leaves an active fragment in rabbit immunoglobulin with reduced antigenic properties, but full biological activity (*9, 10*). CHO is carbohydrate part; PCA presumably is the amino-terminal pyrrolidone-2-carboxyl acid residue; AsX is Asp or Asn (aspartic acid or asparagine).

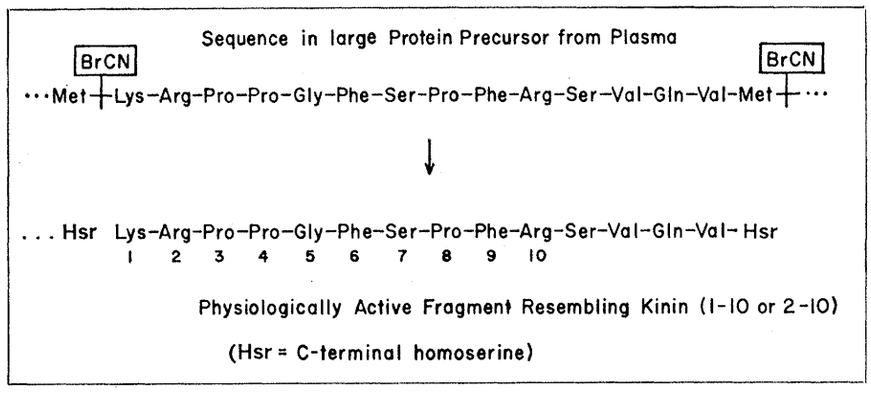


Fig. 3. Scheme of sequences of peptides released from plasma by BrCN.

perature (100°C; 1 hour; 1.0*N* HCl), can also break down by another mechanism, namely, beta elimination to form a dehydroalanine derivative which is easily hydrolyzed to a peptide carboxy-terminal amide and an amino-terminal pyruvic acid peptide. A modification of this reaction is alkylation of the mercapto group by 2,4-dinitrofluorobenzene (*15*) or picryl chloride (*16*).

Treatment with alkali (*p*H 11 to 13) brings about either intramolecular cyclization to the oxazoline (up to 10 percent) or beta elimination to the dehydroalanine which is hydrolytically (or oxidatively) converted to (β-hydroxy)pyruvyl peptide and a peptide amide. The (β-hydroxy)pyruvic acid residue can be removed oxidatively or with *o,o'*-diaminodiphenylamine at *p*H 3 to 6. The application of these methods to the cleavage of reduced cystine bonds in ribonuclease (eight half-cystines) (*17*) and chymotrypsin (ten half-cystines) (*18*) gave the expected seven new amino terminal residues in 30 to 55 percent yield (Tables 2 and 3; Fig. 12).

The alkali-catalyzed bimolecular β-elimination of the disulfide group (*19*) occurs easily in the cystine residues of proteins (*20*) as shown in chart 1. The breakdown of the disulfide (A) is asymmetric (B) (*21*). The direction of the initial C-S bond breakage is governed both by steric factors (*22*) and, in proteins, probably by forces of secondary structure (*23*). The dehydroalanine moiety (C) is capable of adding nucleophilic groups, such as the epsilon-amino group of lysine (*20*), or the delta-amino group of ornithine in alkali-treated wool (*24*), to yield *N*-(2-amino-2-carboxyethyl)lysine (lysinoalanine) (D).

Modifications of the active center of enzymes. When *O*-tosylserine peptides and treated with base, again beta elimination occurs, and a dehydroala-

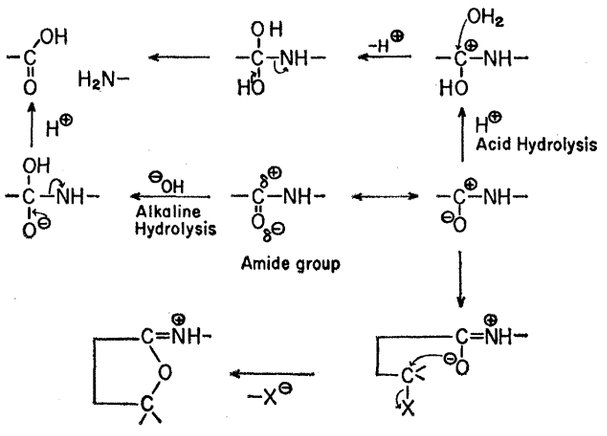


Fig. 4 (left). Hydrolysis of amides by acid, base, and by neighboring group effects.

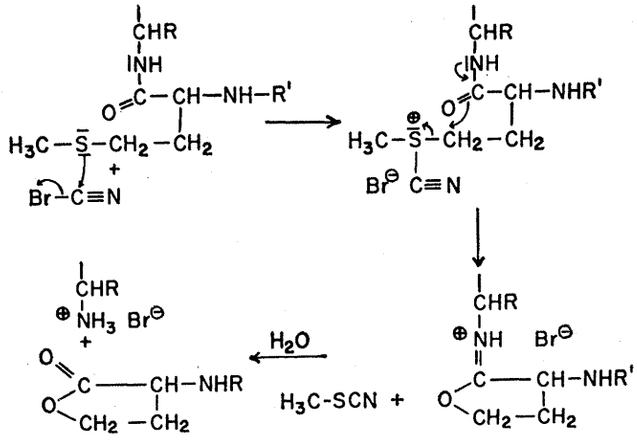


Fig. 5 (right). Mechanism of the cleavage of methionine peptide bonds by cyanogen bromide (*4–6*).

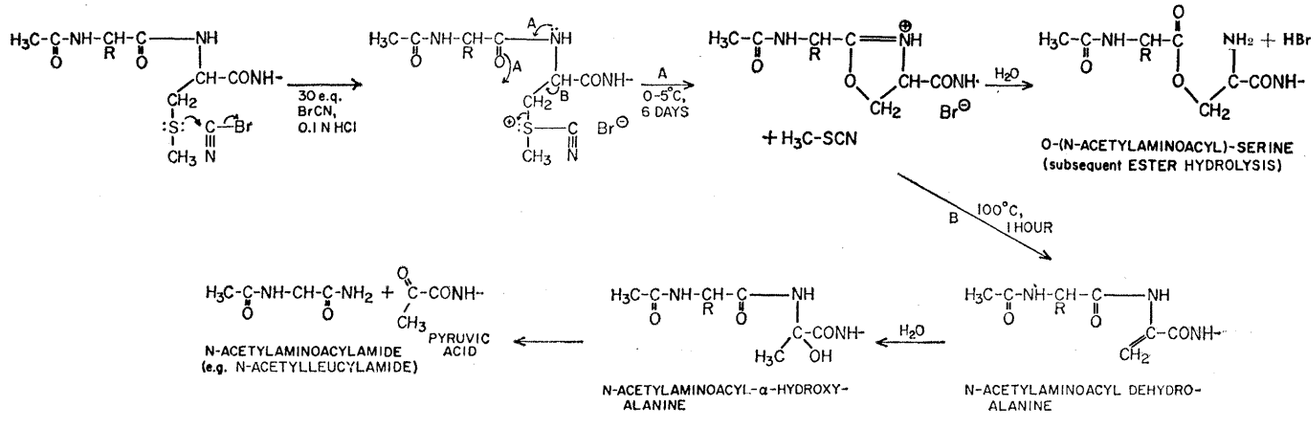


Fig. 6. Mechanism of the cleavage of *S*-methylcysteine peptide bonds by cyanogen bromide at low and elevated temperature (*14*).

Table 3. Cleavage of peptide bonds next to cysteine of modified chymotrypsin. [Degani and Patchornik (*18*)]

Amino acid residue	Position	Yield (mole/mole protein)
Lysine	(170, 205)	0.68
Serine	(225)	0.68
Glycine	(2, 43, 59)	1.65
Alanine	(184)	0.55
Valine	(138)	0.41
Methionine	(195)	0.36*
Isoleucine	(124)	0.33

* Found as methionine sulfone.

nine peptide is formed (*25*) which can be degraded by hydrolysis or oxidation. In this way chymotrypsin was selectively *O*-tosylated at the active serine of the enzyme's catalytic center with tosyl fluoride (chart 2). Base converted this derivative into anhydrochymotrypsin which had lost its enzymatic activity (*26*).

The *O*-tosylate or *O*-mesylate of bound serine can be displaced by other nucleophiles (*27*). This displacement has to be effected cautiously and near neutral *p*H in order to avoid beta elimination and racemization. Thiolacetate at *p*H 5.5 or sodium sulfide (Na_2S) at *p*H 7.3 has been used successfully with the *O*-methanesulfonate of subtilisin, a bacterial protease which lacks disulfide bridges (*28*). The new thiol-subtilisin no longer hydrolyzed ovalbumin or casein (chart 3). It still hydrolyzed *N-trans*-cinnamoylimidazole and certain *p*-nitrophenyl esters.

Differential cleavage with brominating agents. The γ-δ-double bond, although part of an aromatic pyrrole (tryptophan), phenol (tyrosine), or imidazole (histidine) will invite 1,5-interaction of the carboxy carbonyl to form spiro-γ-iminolactones, when a suitable driving force is provided through a bromonium intermediate (positive halogen, *N*-bromosuccinimide, Fig. 7) (*29*). The electronic pull in the case of tyrosine can be exerted through a platinum anode. Continuous-flow electrolysis of proteins is now being studied in order to assess the relative exposure of tyrosine residues to the anode (*30*). The choice of reagents with graded reactivity permits differential cleavage: *N*-bromourea (that is, *N*-bromosuccinimide in 8.0*M* urea) cleaves only tryptophanyl but not tyrosinyl peptide bonds (*31*). Likewise, tribromocresol (2,4,6-tribromo-4-methylcyclohexadienone) in 60 percent acetic acid at *p*H 3 to 4 (pyridine acetate buffer) at 25° or 100°C cleaves only tryptophan, but not tyrosine bonds (*32*). The bromodienone of dibromotyrosine (Fig. 8) is comparable to tribromocresol; it reverts to ethyl 3,5-dibromotyrosinate after donation of its reactive bromine. This pathway of oxidation exists in nature, to judge from the isolation of the bromine-containing antibiotic from *Verongia cauliformis* (*34*), namely 2,6-dibromo-4-acetamido-4-hydroxycyclohexadienone (Fig. 8).

The tribromodienone from tyrosine is only stable as the salt. Above *p*H 7,

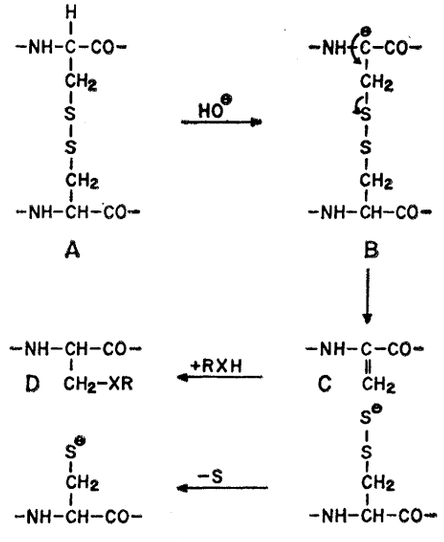


Chart 1

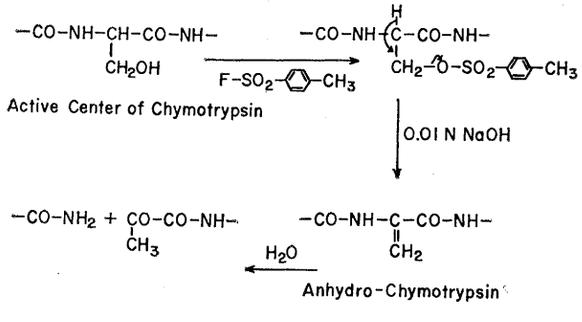


Chart 2

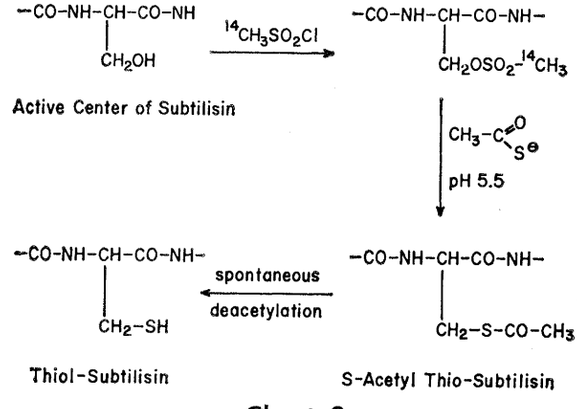


Chart 3

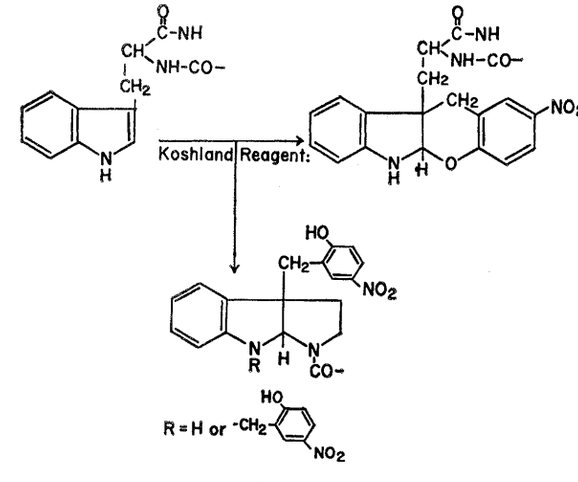
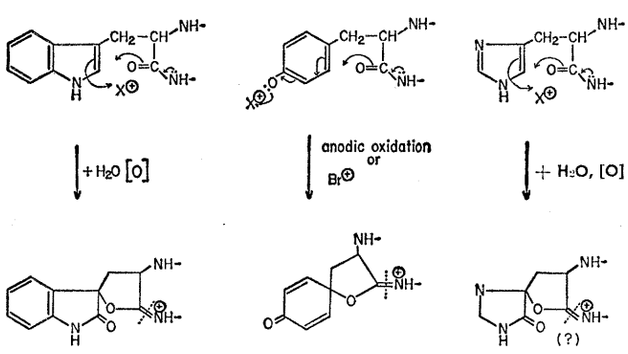


Chart 4



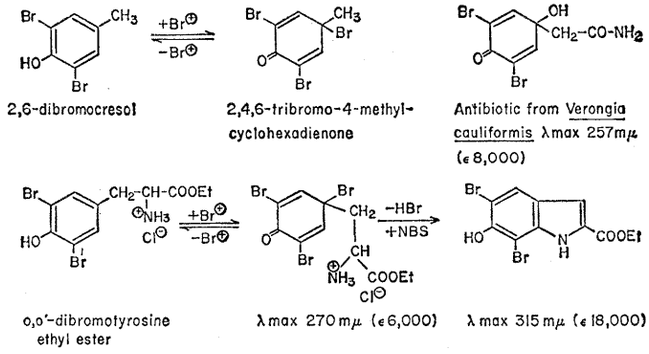


Fig. 7 (left). 1,5-Interactions with the γ,δ- double bonds in the selective cleavage of tryptophan, tyrosine, and histidine peptide bonds by positive halogens. Fig. 8 (right). Synthetic and natural bromo- and hydroxycyclohexadienones from *p*-substituted phenols or tyrosine derivatives.

it spontaneously cyclizes to a 6-hydroxyindole derivative with a characteristic absorption maximum at 315 nanometers. This absorption has been utilized for the rapid assay of amino-terminal tyrosine residues in tryptic digests of proteins (Fig. 9) (*35*).

Another selective reagent for bound tryptophan is the Koshland reagent 2-hydroxy-5-nitrobenzyl bromide (*36*) (chart 4). When peptides and proteins are treated with this reagent—to judge from model studies—tryptophan residues are selectively converted to tetracyclic indoline derivatives (*37*) which are no longer cleaved by *N*-bromosuccinimide (*38*).

Reactivity of tryptophan units in proteins. Depending on the *p*H, bound tryptophan is oxidized both to oxindolealanine and, if cleavage occurs, to dioxindolealanine. Higher than *p*H 9.2, the model compound *N*-acetyltryptophan amide undergoes an interesting intramolecular addition reaction. Initially a short-lived indoline is observed (Fig. 10) (*39*) which, after 30 seconds, rearranges to a new compound with an absorption maximum at 308 nanometers. This new indole has now been identified as a pyrrolinoindole (*40*). This type of oxidation has its analog in nature; sporidesmin is a β-hydroxyeseroline derivative and can be dehydrated to the comparable pyrrolinoindole (*41*). Whether this type of transformation can occur in peptides and proteins or whether, for example, an epsilon-amino group from lysine can displace the bromine of the presumed β-bromoindoline intermediate is still not known.

This difference in the mode of oxidation is probably one of the reasons for the observation that the *N*-bromosuccinimide "titration" of tryptophan in proteins is dependent on many factors, such as *p*H, the nature and concentration of the buffer, the concentration of the reactants and the molar

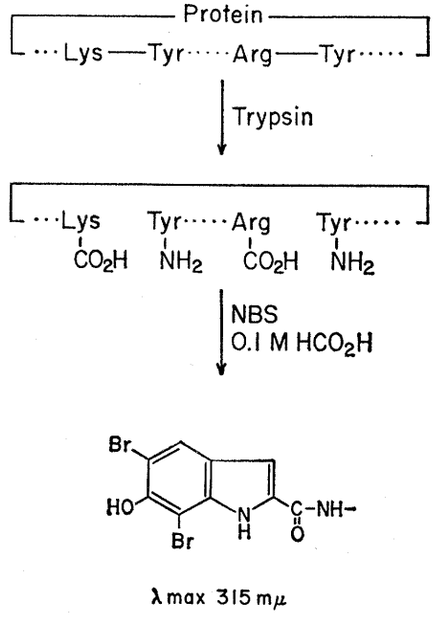


Fig. 9. Rapid assay of peptides with an amino-terminal tyrosine from the tryptic digestion of proteins (*35*).

Table 4. Correlation of chemical reactivity, tertiary structure, and enzymatic activity in hen's egg lysozyme. DBX, α,α'-dibromo-*p*-xylylenesulfonic acid.

Reagent	Selective action	Activity lost	Reference
DBX	Cross-linking of ϵ-NH_2 of Lys: a. Lys(33) to Lys(116) b. Lys(96) to Lys(97)		(*75*)
Phenol-2,4-disulfonyl chloride	Like DBX		(*76*)
N-Bromosuccinimide (NBS)	Try(62) → oxindole	99%	(*77*)
I (I_3^-), *p*H 5.5	Try(108) → oxindole	99%	(*78*)
Ozone (O_3)	Try(108) → formylkynurenine Try(111) → formylkynurenine	None	(*79*)
Iodination in H_2O or 8*M* urea	a. 2 reactive Tyr → monoiodo- and diiodotyrosine b. 1 unreactive ("buried") Tyr is iodinated in 8*M* urea c. Concomitant reaction of 1 Try and His		(*80*)
Cyanuric fluoride, *p*H 9.7	Of three Tyr, two react; unreactive Tyr identical with that of abnormally high pK_a Tyr-53		(*81*)
2-Hydroxy-5-nitrobenzylbromide	Alkylation of 2 Try: Alkylation of 6 Try:	(Very little) 90%	(*82*)
H_2O_2, dioxane-water, bicarbonate, *p*H 8.4	Oxidation product(s) unknown; One out of 6 Try less reactive	95% after oxidation of 1.4 Try	(*83*)

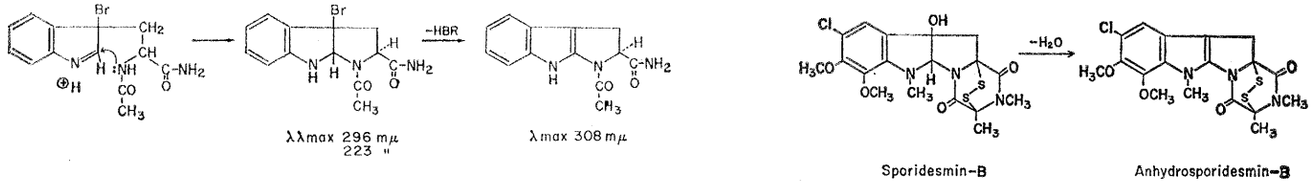


Fig. 10. Natural and synthetic β-hydroxyindolines and pyrrolinoindoles derived from tryptophan (*40*).

ratio of oxidant in relation to protein. Changes in *p*H will also affect and even invert the normally greater reactivity of tryptophan compared with the normally slower-reacting tyrosyl or histidine units. The specificity of the *N*-bromosuccinimide oxidation will probably vary with the environmental factors and the secondary structure characteristic of each individual protein. Thus, this oxidation of lysozyme at *p*H 4 and 5.5 leads to extensive destruction of tyrosine and histidine and to noticeable conformational changes (*42*).

Protection of tryptophan in complexes. Small molecules may interact with proteins in such a way that the conformation as well as the interaction of the protein with a third molecule is altered. There are many variations of this important principle, which is called allosteric effect, especially when it is involved in the dynamics of activation of enzymes in living systems. Sometimes there is no major conformational change. This is the case when the protein avidin (with four subunits, and a molecular weight of 71,000) binds 4 moles of biotin (molecular weight, 244) with the binding energy being approximately that of a covalent bond (*43*). In each subunit there are four tryptophan units, two of which are probably directly involved in, and protected by, binding with biotin and two of which, though not involved in binding, are protected. In this case, the "protection" is from the attack of *N*-bromosuccinimide, which selectively oxidizes bound tryptophan. This protection against attack by *N*-bromosuccinimide has also been noticed in the complex of trypsin with the inhibitor from bovine pancreas (PTI, pancreatic trypsin inhibitor), a tryptophan-free protein with a molecular weight of 6500 (*44*). The reactivity toward *N*-bromosuccinimide may not only serve, within the limitations specified, as a criterion for buried and exposed tryptophan residues in proteins, but also for the involvement of tryptophan in binding sites of enzymes and in enzyme-inhibitor complexes.

Reactivity, enzymatic activity, and tertiary structure of proteins. In a tightly packed three-dimensional protein molecule not all reactive units are equally accessible. Some tryptophan or tyrosine units are "exposed" and chemically reactive, others are "buried" and fail to react. Ionization constants and iodination of tyrosine residues, oxidation, and solvent perturbation of tryptophan residues have been utilized in order to differentiate between exposed and buried residues. The validity of these determinations can be checked, once the complete three-dimensional structure of a protein has been established by x-ray crystallography.

The architecture of the lysozyme molecule has recently become known (Fig. 10) (*45*). Here is an instructive

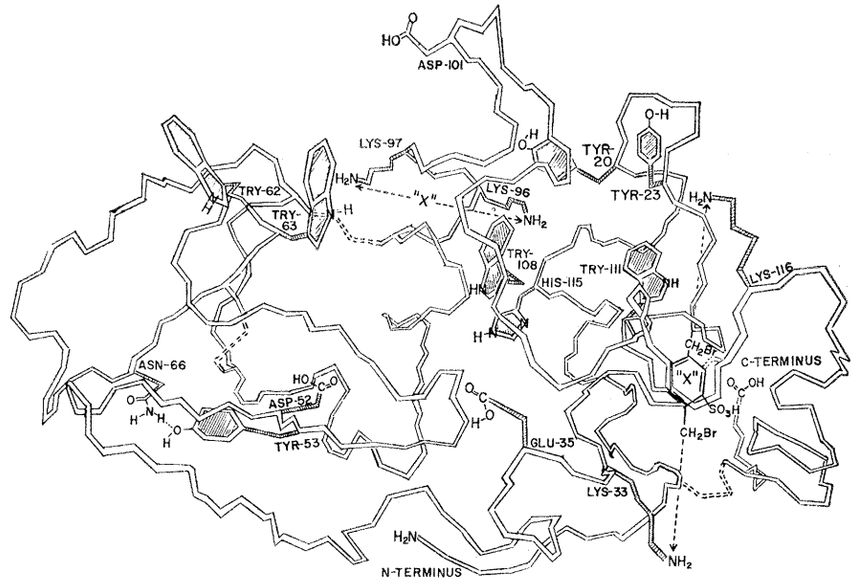


Fig. 11. The tertiary structure of hen's egg lysozyme (*45*).

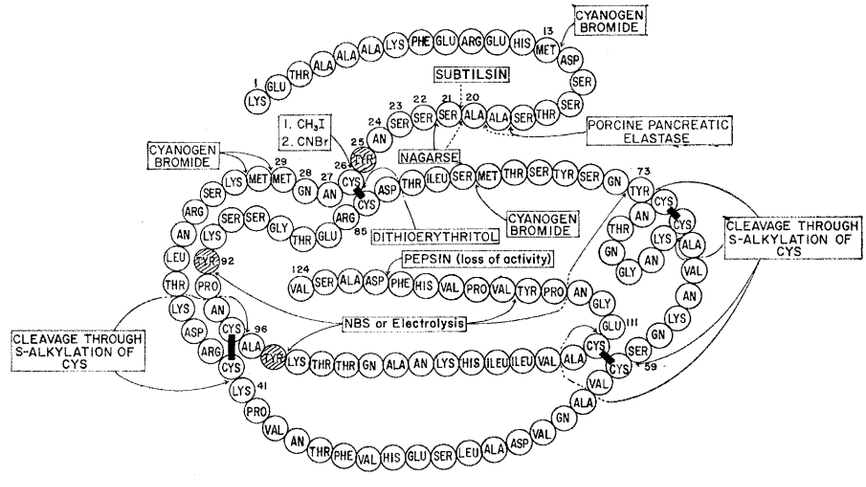


Fig. 12. Schematic representative of the primary structure of bovine pancreatic ribonuclease A, and some of the enzymatic and nonenzymatic cleavage and modification reactions.

example which illustrates how the primary and secondary structure of a protein, as well as the nature of the solvent and oxidant, affect the behavior of reactive residues and, in turn, the catalytic activity of the enzyme.

N-Bromosuccinimide in acetate buffer, *p*H 4.5, according to Funatsu *et al.*, selectively oxidizes Try(62) (Table 4) with substantial loss of enzymatic activity. Oxidation with iodine in aqueous buffer at *p*H 5.5 selectively oxidizes Try(108), with complete loss of activity (*46*). When lysozyme is ozonized in anhydrous formic acid, the tryptophan residues 108 and 111 are selectively oxidized to formylkynurenine residues with retention of full enzymatic activity. Even with the full knowledge of the tertiary structure, these results are difficult to interpret merely in terms of buried or exposed residues. The most exposed tryptophan in the native enzyme is Try(62). Both Try(108) and Try(111), though they may normally be buried in the interior of the native lysozyme molecule, may become more accessible in anhydrous formic acid, a solvent which causes conformational changes in proteins. The two tryptophan residues at 108 and 111 per se are not essential for the maintenance of biological activity. At least the indole rings can be substituted by *N*-formylanthranylyl groups without loss of binding or lytic activity. The reactivity of Try(108) may be due either to the formation of a suitable *N*-iodo intermediate or to an increase of the electron density of the pyrrole ring of Try(108) as a result of interaction with the δ-carboxyl of Glu(35). Oxindolylalanine(108) is apparently unable to substitute for Try(108). Binding of lysozyme with the inhibitor 2-acetamido-2-deoxy-D-glucose protects the protein, that is, especially Try(108), from iodine oxidation. The three tryptophan residues at 62, 63, and 108 have been implicated in the binding site of lysozyme.

This example, and the other chemical modifications listed in Table 4, show that chemical reactivity alone is an erratic guide for probing the tertiary structure of proteins, and that appropriate physicochemical methods, such as ORD (optical rotatory dispersion), solvent perturbation, titrimetry, gel filtration, sedimentation equilibria, are superior in this respect. These methods in any case should be used to ascertain that no gross changes in tertiary structure arise as a result of the chemical modification. In favorable cases, these structural conclusions reached by reactivity studies have been consistent with those obtained by physicochemical methods. Thus, the tyrosines at 73, 76, and 115 in the enzyme ribonuclease are easily iodinated to mono- and diiodotyrosine residues, while the shaded tyrosine residues at 25, 92, and 97 are unreactive and titrate abnormally (Fig. 12). Two out of the three unreactive tyrosines have been shown by x-ray crystallography (*47*) to be hydrogen-bonded to unreactive aspartic acid residues (*48*).

Summary. The sequence of amino acids in large proteins can only be determined when a selective limited nonenzymatic cleavage, for instance by cyanogen bromide, leads to smaller fragments which are separable by column chromatography and amenable to individual enzymatic digestion. The principle of intramolecular participation of amide groups and 1,5-interaction between the carbonyl dipole and a (potential) cationic center has been developed into cleavage and modification of methionine, cystine, cysteine, serine, tryptophan, tyrosine, and histidine peptides. Whereas knowledge of the tertiary structure of a protein permits an assessment of the chemical reactivity of certain functional groups, the opposite method, prediction of tertiary structure from chemical reactivity, works only if the chemical modification does not entail a change of protein conformation.

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Man's Movement and His City

Cities are systems created by man's need and ability to move.

C. A. Doxiadis

Man's relationship, physical and metaphysical, to his city has been interpreted in many ways, successfully and unsuccessfully. Here I am concerned mainly with the relationship of man's movement to his city. It is necessary to understand this relationship at any time, but especially today, when our cities are in a major crisis.

If we fly over any city today, we will see it expanding along the highways or railway lines and canals, and we will probably say that the systems of transportation have a great influence on the cities. That is true. But we sometimes unjustifiably view our cities mainly in terms of systems of transportation. We see that the cities suffer from congestion, and we imagine that, by creating new highways, we will be able to take the very great pressures of traffic out of our cities. Some people even believe that, by solving the problems of transportation in this way, we can solve the problems of the cities.

Such reasoning is only partially correct and leads to invalid conclusions. If we view the problems of our cities only as problems of transportation, then we cannot help the cities, because transportation is only one of many factors and any fundamental solutions must involve them all. Let me give an analogy. The basic unit of the city can be described as a molecule with five elements or "atoms": nature, the original natural environment of man; man, who evolved in nature; society, formed by man and seen here as the system of relationships between men, which may work for or against the interests and values of man; shells, all types of structures and other buildings created by man; and the networks, or systems of transportation, of power, of water supply, of telecommunications, and so on. If we break this molecule, we no longer have a city. However, it is sometimes helpful to consider one element apart from the others in an effort to understand the problem in all its aspects.

The systems of transportation are only a part of one of the atoms or elements of the city molecule—the networks—and this has to be understood before we can proceed. The very term *transportation* may be misleading, since our real interest is man's movement. We tend to forget man's natural movement, based on his own forces, and this is why we have lost the human scale today in our cities. We do not allow people to walk, we "transport" them; we do not allow our children to grow normally; we leave ourselves no room in which to move. We should think of the networks of the city molecule as having four parts: the movement of man, of goods, of power, and of information. "Transportation" refers to the first of these.

Also, before we proceed, we must understand the two basic notions *man* and *his city*, and then try to connect them. We must understand man because he is our main client, and his satisfaction is our main goal; it is for him we are working. Then we must understand the city he builds, since most of his life is spent within it. Only then can we understand man's movement, which connects him with his city.

An understanding of man requires the ability to see him not only as our eyes see him—that is, as a body, or a body plus clothing—but as a system of concentric spheres [in Edward Hall's very apt concept (*1*)]. The system starts with a sphere representing what man sees, but this sphere expands to include what he smells or hears, and expands again to include the space that his mind encompasses, and again to include the space that his soul or psyche encompasses.

An understanding of the city of today requires the ability to see it as a complex system made up of the five units noted above and differing in two

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