PEPTIDE AND PROTEIN LABELLING WITH IODINE. IODINE MONOCHLORIDE REACTION WITH AQUEOUS SOLUTION OF L-TYROSINE, L-HISTIDINE, L-HISTIDINE-PEPTIDES, AND HIS EFFECT ON SOME SIMPLE DISULFIDE BRIDGES.

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Received on July 2, 1973

SUMMARY

Iodinated peptides or proteins (^{125}I*,* ^{131}I*) are used as tracers for radioimmuno assays and for detection in biological systems. The labelling takes place on the aromatic side chains.*
However, when disulfide bridges are present, iodination must be carried out under conditions preventing their degradation. The rate of iodination of L-tyrosine, L-histidine and L-histidine containing peptides by iodine monochloride (ICI) are studied by spectroscopy. Free hitidine increases the hydrolysis rate of aqueous solution of ICI. The interactions between ICI and some simple disulfide are investigated by circular dichroism measurements. These reactions are presented as models for degradation of disulfide bridges in peptide or protein during the iodination process.

INTRODUCTION

The labelling of peptides and proteins has been extensively studied. One approach consists in binding iodine atom(s) onto the aromatic side chains. The iodinated material is then either used as such, the iodine is the labelled, as in the case for instance of radioimmunoassays (1), or is further processed, allowing catalytic replacement of the carbon-bound iodine by tritium. This sequence of reaction permitted the preparation of a variety of tritium labelled, biologicaly active, peptides. (2)(3)(4)(5)(6)(7)(8)(9).

The reaction of iodine or ICl with peptides containing free and accessible thiol groups leads certainly to their oxidation (10)(11)(12). When only disulfide bridges are present, side reactions might also be observed, since iodine can oxidize disulfide bridges, as reviewed by Ramachandran (13), and alter structural characteristics of peptides and proteins in the course of their labelling. The first direct step of iodine reaction with disulfide bridges seems to lead to a charge transfer complex as suggested by crystallographic studies on dibenzyl sulfide iodine complex carried out by Hassel (14) as well as by considerations on molecular orbitals made by Mc. Glynn et al (15). Subsequent hydrolysis of the sulfenyl iodide derivatives produces sulfenic acid (-S-OH) then sulfinic acid ($-SO_2H$) and the disulfide bridge is split.

It is very likely that the positive iodine ion, I^+, issued from iodine monochloride, might behave like iodine. Nevertheless, in practice, ICl appeared less harmful than iodine towards peptidic disulfide bridges as shown by Koshland et al. (16) and by our own experience with different disulfide containing peptides like ocytocin (3), lysine vasopressin (5) or α toxin of naja nigricollis (7).

In order to understand this behavior and to select optimal iodination conditions for labelling purposes, we investigated the reaction of ICl with L-tyrosine, L-histidine and some model peptides, on one hand and its reaction with some disulfides, on the other hand. In both cases, we used spectrophotometric techniques, to monitor the parameters we were interested in, namely the formation of free iodine or iodotyrosine, and the alteration of the disulfide bridges.

MATERIAL AND METHODS

L-alanine, L-phenyl alanine, L-histidine, L-tyrosine and its monoiodo and diiodo derivatives were purchased from Fluka. His-gly ; His-glu ; His-pro-phe ; Val-his-pro-phe ; Ile-his-pro-phe, were gift from Dr. R. Smeby, Cleveland Clinic, Cleveland (Ohio) ; Pyro-glu-his-pro-amide was a gift from Dr. R. Studer, Hofmann-Laroche, (Basel). These compounds were dissolved in 0.1M buffer at a concentration 4 mM, except L-tyrosine which was 1 mM. Sodium formate, sodium phosphate, and ammonium formate respectively, were used for preparing the buffers at pH 4, 7.4 and 9.5. Iodine monochloride (Merck) stock solution was 2 mM in anhydrous methanol.

The kinetic measurements were performed in 1 cm quartz cell equipped with a magnetic stirrer. To 2 ml of buffered solution, aliquots of ICl solution were rapidly added. The molar ratio of amino acid or peptide to ICl was 4 : 1. The iodine formed was monitored at 288 nm as a function of time with a Beckman-Kintrac VII spectrophotometer.

Iodination of L-tyrosine, of L-histidine and of Pyro-glu-his-pro-amide was followed by spectroscopy and by chromatography respectively.

Butyl disulfide (Eastman Organic Chemicals), was dissolved in anhydrous methanol. The 2 mM stock solution was diluted tenfold with anhydrous methanol just before use.

L-cystine (Hoffmann-La Roche) was kept as 1 mM solution in 10 mM HCl. The solution was mixed with an equal volume of buffer before running the spectra.

L-cystine disulfoxyde (Calbiochem) was made 1 mM in HCl.

L-homocystine (Calbiochem) was dissolved into 10 mM NaOH, to the concentration of 13 mM. The solution was diluted to 2 mM in Homocystine with buffers before use.

Oxidized glutathione (Calbiochem) was dissolved in 10 mM HCl to the concentration of 20 mM and diluted tenfold with buffers before use.

Iodine monochloride (Merck) 2 mM in anhydrous methanol was diluted five fold with the same solvent before use. Sodium citrate HCl 0.1 M, pH 2, sodium phosphate 0.1 M, pH 7, and sodium borate 0.1 M, pH 8.5 were used to bring the stock solutions to the desired pH.

Circular Dichroism spectra were obtained with the Roussel-Jouan 185 Model II dichrograph, run at room temperature. Base lines were run either with the compound alone, or with the solvent mixed with the relevant amount of ICl.

The maximum wavelength of the spectra were recorded 15 minutes after addition of ICl. Results were given as molar ellipticities, [θ]. From the dichroic optical density $A_L - A_R$, the molar circular dichroism $\Delta\varepsilon$, and therefore the molar ellipticity [θ], can be calculated according to the relations

$$A_L - A_R = (\Delta\varepsilon)\ c\ .\ d$$

$$[\theta] = 3300\ (\Delta\varepsilon)$$

where c is the molar concentration and d the optical path length.

RESULTS

I - ICl reaction of L-tyrosine, L-histidine and some simple L-histidine peptides in aqueous solutions.

When ICl is added to an aqueous buffer, final concentration 1 mM, the onset and the rate of hydrolysis into iodine depends from the pH. The absorbance at 288 nm was used to monitor the fortion of iodine as suggested by Awtrey et al. (17). At pH 4 and 21°C no iodine is detectable before 96 seconds, then it appeared and reached its maximum concentration in the following 4-5 minutes (Figure 1). The amount of iodine present at the maximum was arbitrarily taken as 100 %. Then iodine reacted with water and formic acid ; the half life of the reaction was about 22 minutes at 21°C under the conditions used. It might correspond to the formation of hypoiodide IO^- and of a colourless compound, iodine formate $(H-COO)_3I$, as suggested since 1861 by Schutzenberger in the preparation of iodine acetate (18). When L-tyrosine was present (1 mM) it reacted very rapidly to give monoiodo tyrosine as checked by spectroscopy and by chromatography. The reaction was completed in 12 sec. and no free ICl was left. Under such conditions, it is very likely that disulfide bridges would remain undegraded, as facing vanishing concentration of iodine, its hydrolysis products or those of ICl. Direct proof was afforded by mixing L-tyrosine and oxidized glutathione before addition of an equimolar amount of ICl. After monoiodo-L-tyrosine formation, the glutathione was recovered intact.

In the presence of 4 mM L-alanine or L-phenylalanine, the appearance of free iodine from ICl took place after 70-80 sec., somewhat earlier than in the control. The maximum of iodine liberated remained identical and its rate of formation was similar (Figure 1).

By contrast free L-histidine catalysed hydrolysis of ICl as showed the immediate liberation of iodine. However the total amount of iodine set free was the same as in the control, indicating that neither ICl, nor its hydrolysis products reacted appreciably with L-histidine during the first 2 minutes at pH 4. Then iodine disappeared with a half life of 9 minutes suggesting a slow reaction of iodine with the protonated form of L-histidine. This influence of free L-histidine on the rate of hydrolysis of ICl prompted an examination of L-histidine containing peptides. His-pro-phe acted like L-histidine in every respects. His-gly and His-glu on the other hand differed from His-pro-phe in their effects on ICl hydrolysis, still allowing the observation of a lag time (30 sec.) before the formation of free iodine, as shown in

Figure 1 - Hydrolysis rate of ICl and its reaction rate with L-amino acids and some L-histidine peptides at pH 4 (ICl/a.a or ICl/peptide : 1/4).

The reaction rate is monitored as O.D. at 288 nm function of time on Beckman Kintrac VII spectrophotometer : top - ICl and L-tyrosine with ICl ; middle - ICl and L-amino acids (Ala, Phe, His), L-histidine peptides (1-His-pro-phe, 2-Val-his-pro-phe, 3-Ile-his-pro-phe, 4-His-gly, 5-His-glu, 6-Pyro-glu-his-pro-amide or T.R.F.) with ICl ; bottom : ICl and L-histidine, L-histidine peptides serie (n° is identical as in the middle of the figure) with ICl.

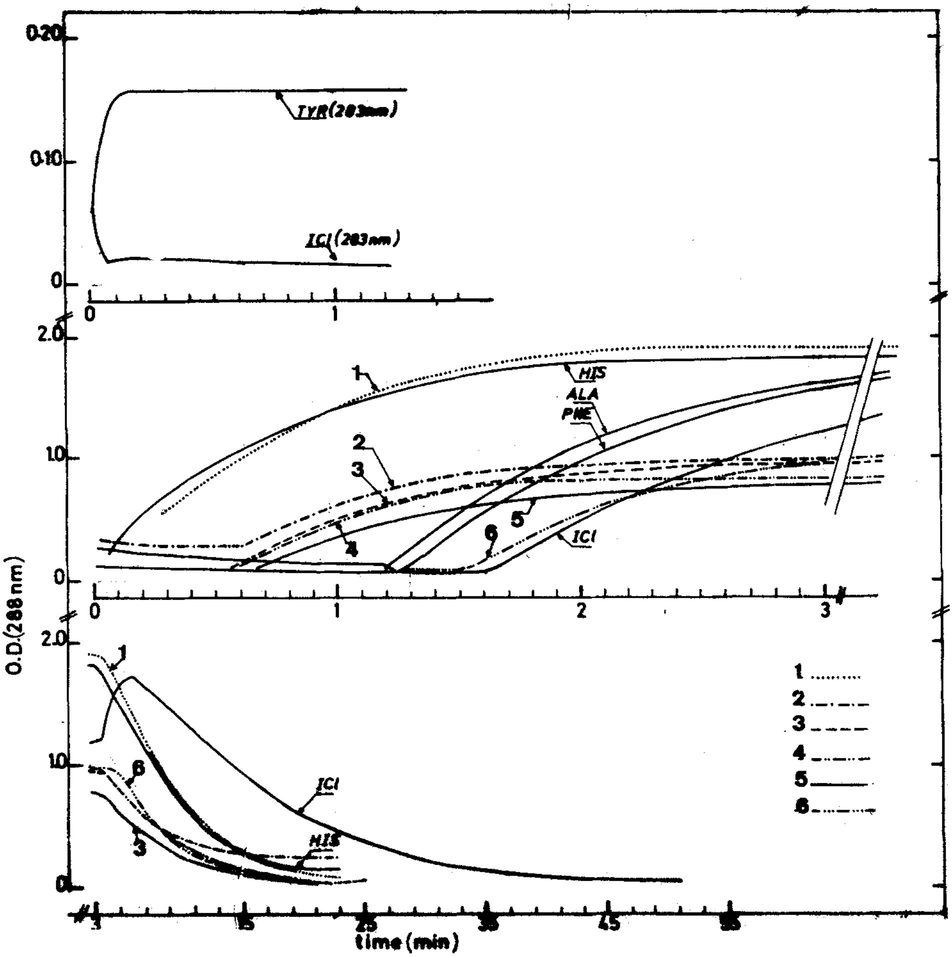
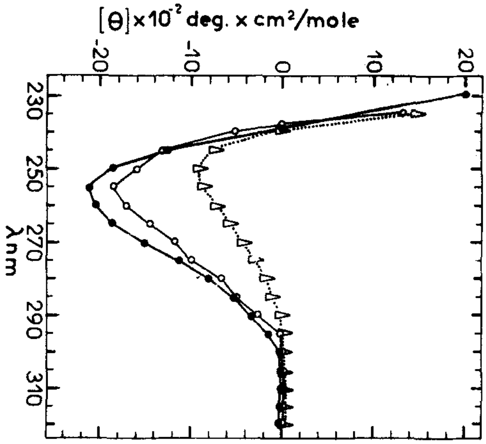


figure 1. Moreover the total amount of iodine liberated was found less than in the control, suggesting a reaction of the peptide either with ICl or with its primary products of hydrolysis. Their reaction rate with free iodine remained in the same range as observed with L-histidine or His-pro-phe, since the half life of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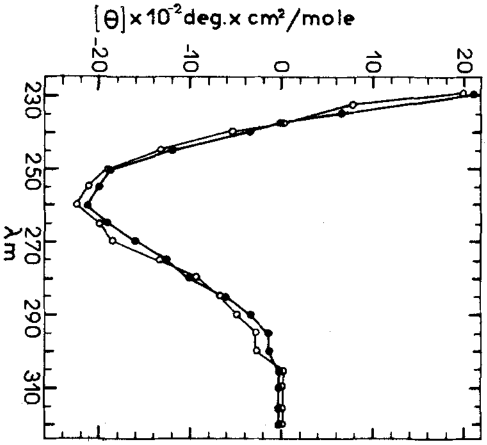
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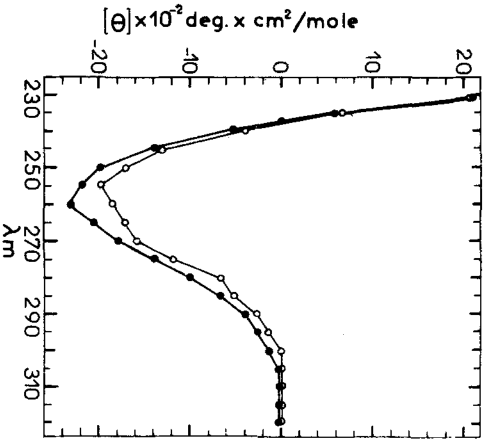
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Figure 2 - CD spectra in the 230-320 nm region of L-cystine and L-cystine with ICl at various pH : left-(-•-•-) L-cystine, (-o-o-) L-cystine with ICl and (..▲...▲..) L-cystine disulfoxide at pH = 2; middle-(-•-•-) L-cystine and (-o-o-) L-cystine with ICl at pH 7 ; right-(-•-•-) L-cystine and (-o-o-) L-cystine with ICl at pH = 8.5 (Ratio I/-S-S- = 2).

iodine disappearance was also 10 minutes. Val-his-pro-phe or Ile-his-pro-phe led to a similar profile as His-gly. But most striking is the effect of the hormone Pyro-glu-his-pro-amide ; it did not promote the formation of free iodine and reacted with ICl as shown by the curve of figure 1 and by the isolation of the iodo derivative (6) ; these findings indicated that the conformation in solution of the peptide mentioned, and more specially the surroundings and the degree of freedom of the L-histidine side chain played a role in the catalytic influence of the imidazole ring on ICl hydrolysis. That Pyro-glu-his-pro-amide, the hypothalamic thyrotropin releasing factor (TRF), presented in solution a specific configuration, has already been established (19) and is supported by the present data. The remarks we wished to make here concerned the possible side reactions following iodination with ICl at pH 4. As a function of the peptide structure at hand, ICl either reacted directly with the aromatic side chain or was hydrolysed leading to the formation of free iodine.

At pH 7.4, the rates of the primary reactions taking place after addition of ICl in an aqueous buffer were increased, leading to the undelayed appearance of free iodine. Its rate of formation, however is the same than at pH 4. At pH 7.4 L-tyrosine reacted in less than 10 sec. with ICl, and allowed no detection of free iodine.

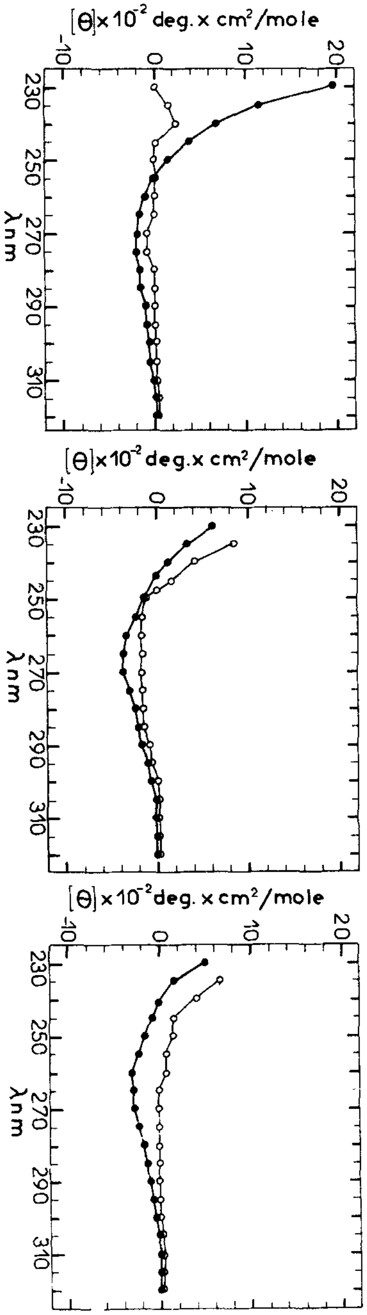
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Figure 3 - CD spectra in the 230-320 nm region of L-homocystine : (-•-•-) L-homocystine and (-o-o-) L-homocystine with ICl at various pH (left : pH = 2 ; middle pH = 7 ; right pH = 8.5). (Ratio I/-S-S- = 2).

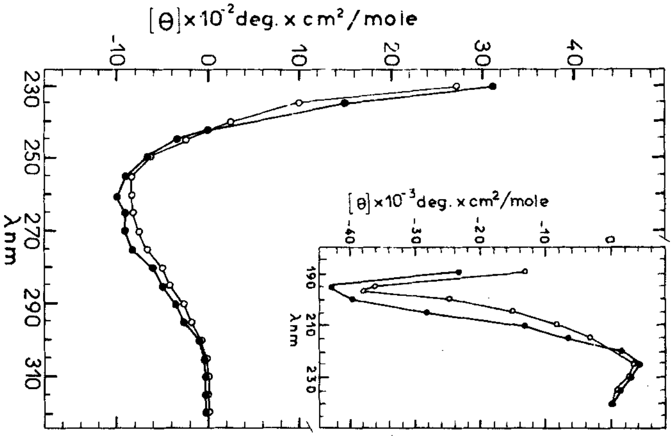
By contrast with pH 4, L-histidine was iodinated as rapidly as L-tyrosine at pH 7.4 and no free iodine could be detected. At pH 9.5, as expected, ICl and free iodine did react so rapidly with water that iodine did not accumulate. Free L-tyrosine and L-histidine were iodinated in less than 10 seconds.

In view of these results showing an increased reactivity of ICl towards L-histidine but not towards L-tyrosine when the pH is raised from 4 to 7.4, we were led to consider the fate of disulfide linkages.

II - Effects of ICl and Iodine on disulfide bridges.

Disulfide linkages present electronic transitions of the $n \rightarrow \sigma^*$ type and possess in general two characteristic absorption bands, for instance at 194 nm and 250 nm, in the case of ethyl disulfide (20). For this reason, we carried out CD measurements around 250 nm ; the CD spectra obtained at various pH for L-cystine, L-homocystine and oxidized glutathione are reported in figure 2-4. A minimum is observed between 250-270 nm, the small-

Figure 4 - CD spectra in the 230-320 nm region and 185-240 nm region of Oxidized glutathione : (-•-•-) Oxidized glutathione and (-o-o-) Oxidized glutathione with ICl at various pH (left : pH = 2 ; middle pH = 7 ; right pH = 8.5). (Ratio I/-S-S- = 2).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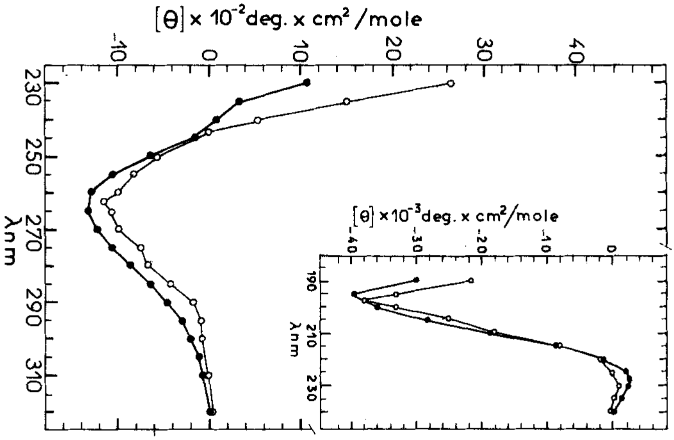
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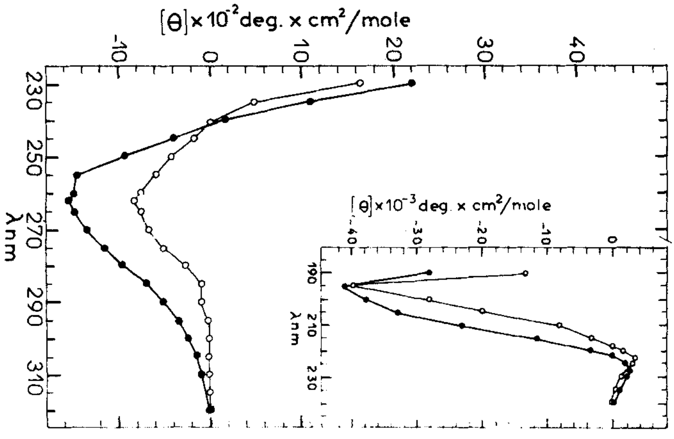
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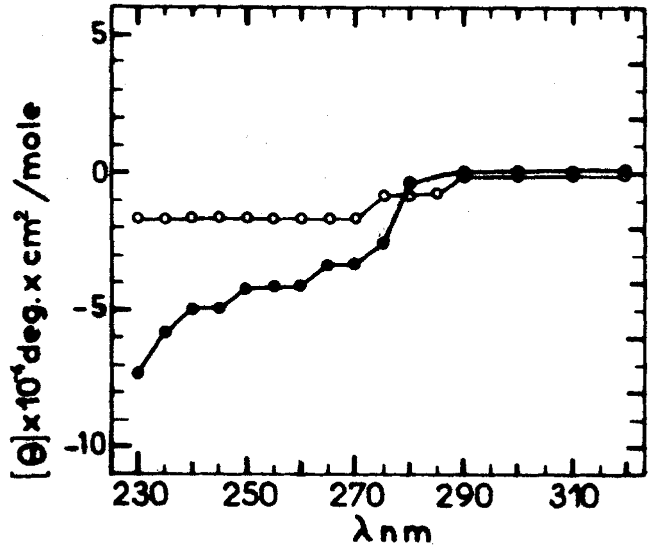
est negative ellipticity being afforded by L-homocystine, in agreement with Beychok's report (21). Butyl disulfide spectrum presented no minimum in the wavelength range studied (Figure 5).

The molar ellipticities in the region of λ max of the spectra obtained 15 minutes after addition of ICl were always different from the reference spectrum, when the experiments were run at pH = 8.5. A reduction in ellipticity was observed, indicating a modification in the electronic transition of the sulfur atoms. At neutral pH, L-cystine appeared to be unaffected by ICl under the condition used. By contrast oxidized glutathione, presented a reduction of ellipticity between 250-290 nm and L-homocystine a relatively much larger alteration. At acidic pH, L-cystine, oxidized glutathione and L-homocystine were altered, and the latter to a large extent. It should be noted that at pH 7 and below, the iodine formed by hydrolysis of ICl remained present during the course of the experiment, except in the presence of L-homocystine. The stoechiometry I/S-S was 2, the disappearance of the yellow coloration due to iodine suggested an extensive reaction of I with the disulfide of L-homocystine. Finally, butyl disulfide CD spectrum was quite extensively modified by addition of ICl, in the single experimental condition used, a solution in methanol (Figure 5).

The reduction in ellipticity observed corresponded very likely to binding of iodine to the sulfur by charge transfer. This binding is expected to reduce the degree of freedom of the p doublets of the sulfur, and thus alter the $n\text{-}\sigma^*$ transition of the S-S linkage. A similar effect is produced when oxygen atoms are bound to a disulfide as for instance in L-cystine disulfoxide. Figure 1 shows that the ellipticity if cystine disulfoxide is

Figure 5 - CD spectra in the 230-320 nm region of Butyl disulfide : (-•-•-) Butyl disulfide in anhydrous methanol and (-o-o-) Butyl disulfide with ICl in the same solvent. (Ratio I/-S-S- = 2).



reduced compared to that of L-cystine, in the range of wavelengths studied.

A comparison of the alterations provoked by ICl and its hydrolysis products is made possible by the difference (θ)control - (θ)+ICl , plotted as percent if (θ) control in the region 250 - 270 nm (table 1). It appears thus that butyl disulfide and L-homocystine were more reactive towards ICl than L-cystine and oxidized glutathione. This behavior might reflect structural characteristics. The energy barrier associated with a rotation of a C-C linkages is of the order of 2 - 3 Cal/bond whereas it reaches

10-12 Cal/bond for a S-S linkage (22). Rotation is thus restricted for the latter, which, in the absence of the other constraints, as in ethyl disulfide leads to a preferred dihedral angle C_1-S-S, S-S-C_2 of about 90° (23).

TABLE 1

ALTERATION OF THE MOLAR ELLIPTICITY OF VARIOUS DISULFIDES IN THE PRESENCE OF IODINE MONOCHLORIDE (RATIO I/-S-S- = 2)

Product		$\Delta\theta$ = (θ) control − (θ) +ICl deg x cm^2/mole	$\frac{\Delta\theta}{\theta}$ % control
Butyl disulfide		25 000 (250 nm)	61
	pH = 2	250 (255 nm)	12
L-cystine	pH = 7	120 (260 nm)	5
	pH = 8.5	300 (260 nm)	13
	pH = 2	120 (270 nm)	60
L-homocystine	pH = 7	220 (265 nm)	57
	pH = 8.5	380 (260 nm)	126
Oxidized	pH = 2	160 (260 nm)	16
Glutathione	pH = 7	200 (262 nm)	15
	pH = 8.5	730 (262 nm)	47

In the amino acids disulfide however, intramolecular bonding by hydrogen or electrostatic bonds are stabilizing one rotamer, but to a different extent according to the molecule considered. Certainly L-homocystine, in this respect, is more flexible than L-cystine, as the squeletton of the former contain one more carbon atom in each moiety.

The reactivity of the sulfur seems thus to be in relation with the degree of stabilization of a given rotamer. Another exemple is afforded by the reaction rate of silver nitrate, which as shown by Cecil et al. (24), decreased as 2400, 28, 9 when the disulfide was L-homocystine, oxidized glutathione and L-cystine respectively. It is interesting to note that the extent of reduction of the ellipticity after ICl addition followed the same order.

Butyl disulfide, which lacks intramolecular stabilizing bonds reacted extensively with ICl, ressembling L-homocystine. The pH effect noted in table 1 might thus be a combination of its influence on the stability of intramolecular linkages as well as on the rate of reaction of ICl and iodine.

CONCLUSION

The labelling of peptides or proteins with iodine at the aromatic side chains of L-tyrosine and L-histidine might encounter difficulties when the molecule contains oxidizable components. In view of the fact that the iodinating reagent is facing two partners, the aromatic rings and the disulfide bridges, one has to select the conditions which confers a faster reaction rate to the aromatic rings. In this respect, ICl has been shown to react very rapidly with L-tyrosine at pH = 4 and with L-tyrosine or L-histidine at neutral pH, allowing iodination in a matter of seconds. It thus appears favorable pratically to carry out iodination by successive addition of small aliquots of ICl. The second onclusion refers to the influence of the structure both on the ate of ICl hydrolysis and on the reactivity of the disulfide ridges.

In both cases different surrounding structures conferred ifferent behaviors to the same group, the imidazole ring or he disulfide. It might be inferred that in peptides or in proteins similar alteration in reactivity ought to be encountered, nd more specifically that in certain cases, disulfide bridges ight show enhanced susceptibilities. Modifications of the actors which contribute to control the structure of the molecule nder investigation, as temperature, pH, and solvent, associated with CD measurements represent a convenient way to select the optimal iodination conditions. In this respect, to improve the apparent specificity of the iodinating reagent towards aromatic residues, one can consider using a disulfide as protecting agent. From table 1, L-homocystine appears to be a good candidate.

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