destroyed, thereby accounting for specific functional changes. These could be differentiated from nonspecific changes, due to denaturation.

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Iodination of the Normal and Buried Tyrosyl Residues of Lysozyme. I. Chromatographic Analysis*

I. Covelli† and J. Wolff

ABSTRACT: Iodination of lysozyme with ^{131}I-labeled KI_3 solution (up to 14 moles/mole of lysozyme) at pH 8.5, in H_2O or 8 M urea at either 0° or 24°, followed by enzymatic hydrolysis and chromatography revealed that: (1) the rate of iodination of the first two tyrosyl residues was faster in 8 M urea and at 24°. The recovery of organic iodine was greater under these conditions with a maximum of 7 g-atoms of I/molecule of lysozyme. Oxidative side reactions were also maximal under these conditions. (2) Only two of the three tyrosyl residues were iodinated in H_2O, whereas in 8 M urea all three were iodinated. Monoiodotyrosine was quantitatively important only at low levels (<2 moles of I_2/mole of lysozyme) of iodination. Diiodotyrosine was the major product of all three tyrosyl residues but monoiodohistidine (and some diiodohistidine) appeared at molar ratios of $I_2 > 2$, *i.e.*, after one tyrosyl residue was fully iodinated. Thyroxine was not found.

It has been known for some time that the tyrosyl groups of lysozyme ionize with abnormally high pK values. Initially, all three[1] phenolic dissociations were thought to be identical, with an apparent pK value of 10.8 (Fromageot and Schneck, 1950; Tanford and Wagner, 1954; Donovan *et al.*, 1961). However, the titration curves were broader than the expected and the possibility that this was due to appreciably different pK values was entertained (Tanford and Wagner, 1954). At present it seems most probable that lysozyme contains three tyrosyl residues, two of which ionize instantaneously with an apparent pK of 10.4–10.5, while the third ionizes very slowly and with a midpoint in the titration curve at 12.8 (Inada, 1961; Edelhoch and Steiner, 1962). Ionization of this buried tyrosyl residue is time dependent (Inada, 1961). The enzyme is incompletely denatured in 8 M urea, whereas 4 M guanidine consistently causes major configurational changes, making possible titration of several masked

* From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland. *Received October 13, 1965.*

† Recipient of a NATO Fellowship. On leave from the Istituto di Patologia Generale, Università di Napoli, Italy.

[1] Fromageot and Schneck (1950) titrated only two tyrosyl residues and these had a normal titration curve.

residues including the third tyrosine (Tanford and Wagner, 1954; Leonis, 1956; Donovan *et al.*, 1960; Inada, 1961; Edelhoch and Steiner, 1962; Steiner, 1964).

These structural features should be reflected in the ability of the tyrosyl (and histidyl) residues to react with iodine. Fraenkel-Conrat (1950) found, some years ago, that large amounts of iodine caused loss of enzymic activity, which was partly restored by addition of reducing agents early after iodination. Reversibility was ascribed to the histidyl residue which was believed to participate *via* an initially reversible iodination (0.7–0.85 atom/mole) of its nitrogen with a subsequent N–I to C–I rearrangement. Only a fraction of the tyrosyl residues had been iodinated under these conditions.

The present study was initiated in order to investigate thyroxine formation in proteins of known structure. To this end, iodination of native and urea-treated lysozyme has been performed in an attempt to evaluate the reactivity toward the iodine of the iodinatable residues in the protein. While thyroxine was not found, different reactivities of the three tyrosyl and the one histidyl residues toward iodine have been found by chromatographic analysis of the hydrolyzed protein. Spectrophotometric titrations of lysozyme iodinated under various conditions as well as the effects of oxidative side reactions will be considered in the accompanying paper (Wolff and Covelli, 1966).

Experimental Section

Materials. Salt-free 2× crystallized egg white lysozyme was obtained from Worthington Biochemical Corp. (Lot F-635) and used without further purification. The molecular weight was taken as 14,307. The protein was iodinated with triiodide solution (0.046 M I_2 in 0.14 M KI) which was standardized against $AgNO_3$ (Kolthoff and Stenger, 1947), and $Na_2S_2O_3$, using soluble starch as indicator. Microdeterminations of total I (by the Boston Medical Laboratory) according to a modification of the chloric acid method (Benotti and Benotti, 1963) were also carried out as independent checks of the above methods. Iodinating solutions were used for a whole series of iodinations. The triiodide was labeled with $Na^{131}I$ which was free of carrier and reducing agents, and supplied in dilute NaOH (Oak Ridge National Laboratory, Tennessee).

Reagent grade urea was recrystallized from absolute ethanol. Methylmercaptoimidazole was obtained from Eli Lilly & Co; Pronase, B grade from Calbiochem; DIT[2] (3,5-diiodo-L-tyrosine) from Nutritional Biochemical Corp.; MIT (3-monoiodo-L-tyrosine) from the Aldrich Chemical Co.; L-histidine from Eastman Organic Chemicals; Tris (tris(hydroxymethyl)-aminomethane) was of primary standard grade from the Sigma Chemical Co.; MIH (2- or 5-monoiodo-L-histidine) and DIH (2,5-diiodo-L-histidine) were synthesized according to Brunings (1947). All other compounds were of reagent grade. Triply distilled water was used throughout.

Methods. IODINATION PROCEDURE. Lysozyme (20–40 mg) was dissolved in 0.8 ml of water or 10 M urea, and 0.2 ml of 1.0 M Tris–HCl buffer at pH 8.50 was added to make the final concentrations of Tris and urea 0.2 and 8.0 M, respectively. Under these conditions, the pH remained unchanged during the iodination. Iodinations were carried out, at 0° and at room temperature (24°), at two different rates: (a) slow, for the studies on the yields of iodoamino acids; (b) fast, for kinetic studies.

In the slow iodinations, the iodinating solution was added to a magnetically stirred lysozyme solution, at a rate of 0.001 ml/min with a motor driven Agla syringe and a polyethylene catheter immersed in the protein solution. The amounts added were recorded automatically. Usually, iodination times were between 30 and 90 min. When the solutions started to become persistently yellow or cloudy, iodination was suspended for a few minutes. After iodination, the solutions were kept at +4° for 30 min, then 15 or 20 μl of 1.0 M $K_2S_2O_3$ was added to stop iodination and to eliminate any reversible interactions between the iodine and the protein.

In the fast iodination (used in kinetic experiments only) 9 moles of I_2/mole of protein was added directly to the lysozyme solution. At time intervals of 1–180 min, samples were removed after thorough mixing, and put directly into acetylated Visking tubing (0.39-in. flat width) containing an excess of thiosulfate.

DIALYSIS. The iodinated samples were dialyzed in acetylated Visking membranes (Craig and King, 1962) at +4° with external stirring and frequent changes of glass-distilled water for 18–24 hr. At the end of the dialysis, the suspensions were centrifuged at 5000*g* for 15 min. The clear supernatant solutions were used for determinations of radioactivity and protein concentration.

RADIOACTIVITY DETERMINATIONS. ^{131}I content of the supernatant solutions after dialysis were determined to a counting error of <1.0% in a well-type scintillation counter equipped with a spectrometer.

PROTEIN DETERMINATIONS. The protein concentrations of each sample were determined by a biuret method (Gornall *et al.*, 1949) with a standard curve of native lysozyme. This method was chosen in order to avoid dependence on the phenolic function. The protein remaining in the supernatant solutions after iodination varied between 30 and 70%. For weakly iodinated lysozyme a molecular weight of 14,500 was taken and for fully iodinated ones it was assumed to be 15,100. The number of gram-atoms of I bound per mole of protein was calculated from duplicate determinations of the ^{131}I content of the dialyzed protein and the specific activity of the iodinating solution.

ENZYMIC DIGESTION. Twenty microliters of a freshly prepared Pronase solution (20 mg/ml) in 1.0 M Tris–HCl buffer at pH 8.0 plus 0.001 M methylmercaptoimidazole

[2] The following abbreviations are used: MIT, 3-monoiodo-L-tyros(ine)(yl); DIT, 3,5-diiodo-L-tyros(ine)(yl); MIH, 2- or 5-monoiodo-L-histid(ine)(yl); DIH, 2,5-diiodo-L-histid(ine)(yl).

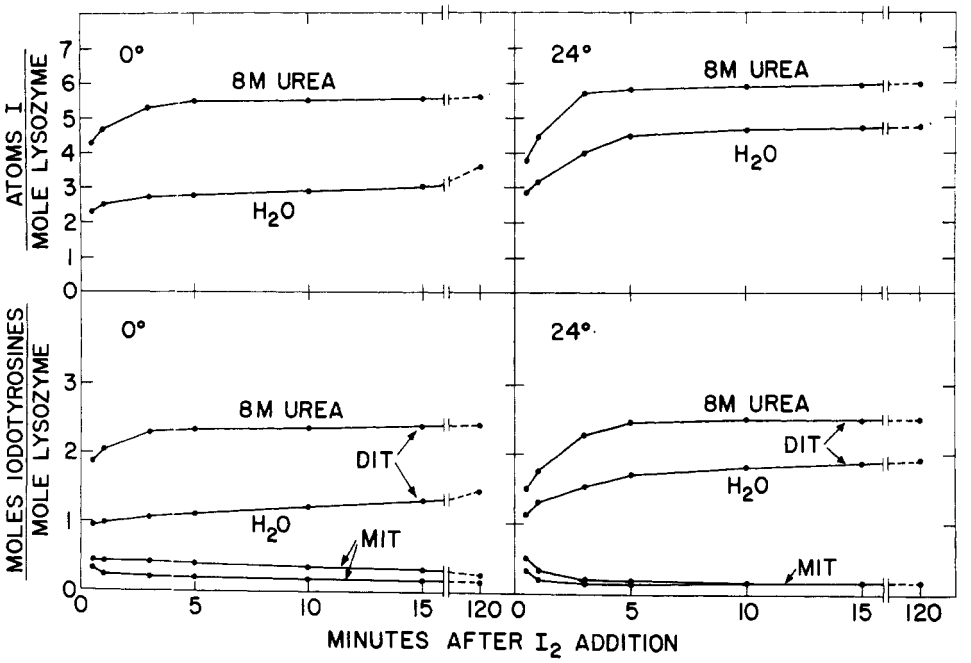


FIGURE 1: Kinetics of iodination of lysozyme at 0 and 24°. Nine moles of I_2 per mole lysozyme was added at zero time. Curves labeled H_2O are in 0.2 M Tris buffer, pH 8.50; curves labeled 8 M urea contain the same buffer. Upper quadrants: Total iodine bound as a function of time as calculated from the radioactivity of the dialyzed preparation and the specific activity of the iodinating solution. Lower quadrants: MIT and DIT formed as a function of time calculated from the total iodine bound and chromatographic analysis of the digested lysozyme.

was added to a sample containing 3–5 mg of protein in about 0.2 ml and allowed to incubate for 5 hr at +37°. Then the tubes were placed on ice and portions of the digests were chromatographed. Preliminary experiments showed that these were the optimal conditions for maximal digestion with minimal deiodination.

PAPER CHROMATOGRAPHY. The chromatographic systems used were: (a) 1-butanol–ethanol–0.5 N ammonia (250:50:100), descending, ~12 hr, Whatman No. 2 paper; (b) 1-butanol saturated with 2 N acetic acid, ascending, 16 hr, Whatman No. 3MM paper; (c) ethanol–1.0 M ammonium acetate (7.5:3), ascending, 16 hr, Whatman No. 3MM paper; (d) pyridine–2 N acetic acid (8:2), ascending, 12 hr, Whatman No. 3MM paper; (e) 2,4,6-collidine–2 N acetic acid (8:2), ascending, 18 hr, Whatman No. 3MM paper. Carriers were identified with Pauly's reagent. Aliquots were chromatographed with or without carrier immediately after application to the paper and drying in a stream of cold air. Radioactivities were recorded in a 4π strip scanner and the areas quantitated by planimetry. Resolution was also checked by radioautography on Ansco No-Screen X-ray film.

Results

Kinetics of Iodination. The rate of organic iodine formation in lysozyme in 0.2 M Tris–HCl buffer or 8 M urea at pH 8.50 and at either 0° or 24° is depicted in Figure 1. The total amount of iodine (9 moles of I_2/mole of protein) was added to start the experiment and suitable aliquots were withdrawn at various intervals. This amount of I_2, although in excess of the total iodinatable positions (equivalent to 8 moles of I_2/mole of lysozyme), was not sufficient to give complete iodination. In water 2 g-atoms of iodine was substituted very rapidly at 0°, while at 24° this number was near 3. Maximum substitution (120 min) in H_2O amounted to ~3.5 and ~5.0 g-atoms at 0° and 24°, respectively. The maximum was attained gradually at 0° but was nearly complete in 5 min at 24°. In 8 M urea, 4 g-atoms of I were rapidly substituted regardless of temperature and the maximum value attained was only slightly higher at 24°, and was reached in 3–5 min at both temperatures.

If the yield of iodinated tyrosine residues is plotted *vs.* time (lower two quadrants of Figure 1) the nature of the change appears to be the same as for the total organic iodine. However, the yield of organically bound iodine was always higher than that recovered as iodine of the iodotyrosyl residues (see below). Note that MIT was a very small fraction of the iodinated tyrosyl residues even at the earliest times.

Recovery of Organically Bound Iodine. The disposition of increasing amounts of iodine added slowly to lysozyme at pH 8.50 and allowed to react for 2 hr in H_2O or 8 M urea at 0° or 24° is detailed in Tables I and II. Under all conditions used, the first 2 moles of I_2 was almost quantitatively converted to organic iodine. Thereafter, increasing amounts of iodine were lost in side reactions, presumably oxidations. Although sub-

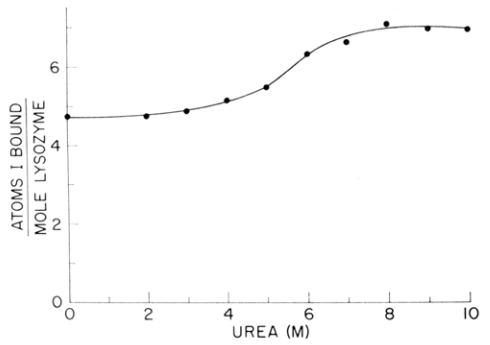


FIGURE 2: Effect of the urea concentration on the iodination of the lysozyme. Iodination at +24°, pH 8.50, with 12.0 moles of I_2/mole of protein, added gradually over 30 min.

TABLE 1: Disposition of Iodine after Iodination of Lysozyme at 0°.

Conditions of Iodination	Moles of I_2 per Mole Lysozyme	Bound[a]	Unreacted[b]	Lost[c]
H_2O	2	2.0	0	0
0°	4	3.2	0.5	0.3
pH 8.5	6	3.4	1.6	1.0
	8	3.6	3.2	1.2
	10	3.8	4.8	1.4
	12	3.9	6.6	1.5
	14	4.0	8.4	1.6
8 M Urea	2	2.0	0	0
0°	4	3.6	0.1	0.3
pH 8.5	6	4.1	1.1	0.8
	8	4.7	1.9	1.4[d]
	10	5.6	2.8	1.6
	12	5.7	4.3	2.0
	14	5.9	5.0	3.1

[a] As calculated from the specific activity of the triiodide solutions. [b] As calculated by titration with $Na_2S_2O_3$. [c] Obtained by difference; I_2 lost = total I_2 added − (I_2 bound + I_2 unreacted). [d] First appearance of a faint yellow color remaining after addition of reducing agent.

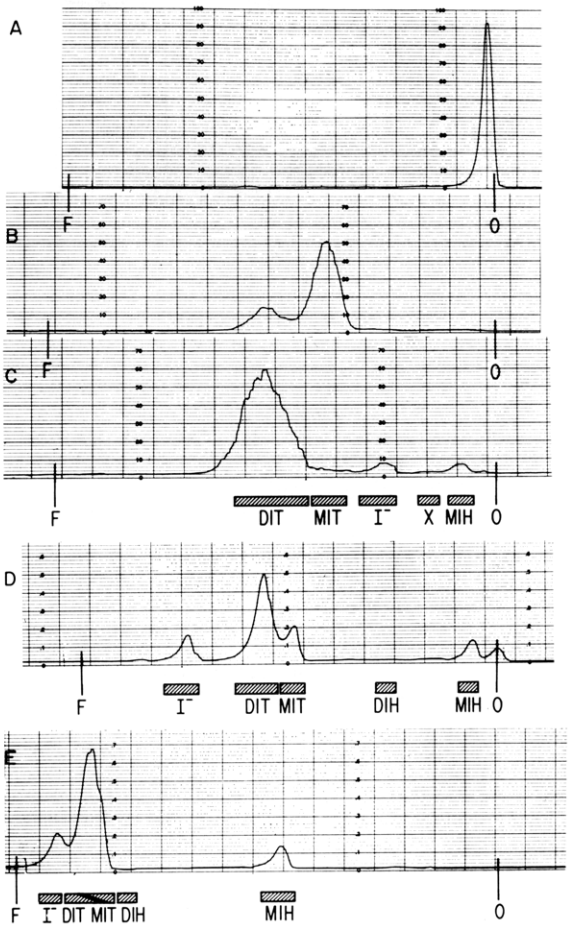


FIGURE 3: Scans of ^{131}I of iodinated lysozyme after prolonged dialysis, without digestion (A) or after digestion with pronase (B–E). (A) Iodination in H_2O, at +24°, with 4.8 moles of I_2/mole of lysozyme; solvent: 1-butanol saturated with 2 N acetic acid. (B) Iodination in H_2O, at +24°, with 0.9 mole of I_2/mole of lysozyme; solvent: 1-butanol saturated with 2 N acetic acid. (C) A, after pronase digestion; solvent: 1-butanol saturated with 2 N acetic acid. (D) Iodination in 8 M urea, at 0°, with 8.0 moles of I_2/mole of lysozyme; solvent: collidine–2 N acetic acid (8:2). (E) D; solvent: pyridine–2 N acetic acid (8:2). The positions of the radioactive spots on the strips were located from the added carrier compounds. 0 = origin; F = solvent front.

stitution attained a maximum of only 4.0 g-atoms of iodine in H_2O at 0°, the amount of I_2 lost by oxidations was held to a minimum and most of the added I_2 was unreacted as shown by the complete disappearance of the yellow color upon reduction as well as by titration for I_2. In 8 M urea at 0°, substitution increased to a maximum of 6 moles, the amount of iodine lost in oxidations more than doubled, and there was residual yellow color after reduction. At 24°, 5 moles of organic iodine was formed in H_2O. This represents an increase of ~1.0 mole over 0°. In addition, about 2.5 times as much I_2 was used for oxidations, leading to a permanent faint yellow color not found at 0°. Maximal substitution and oxidation occurred in 8 M urea at 24°. These reactions were so extensive that no free I_2 was recovered despite the addition of ~50% excess of I_2. The product was, furthermore, markedly yellow. The increase in iodination produced by urea was a function of the urea concentration and did not attain its maximum until ~8 M urea was used (Figure 2). Below 4 M urea

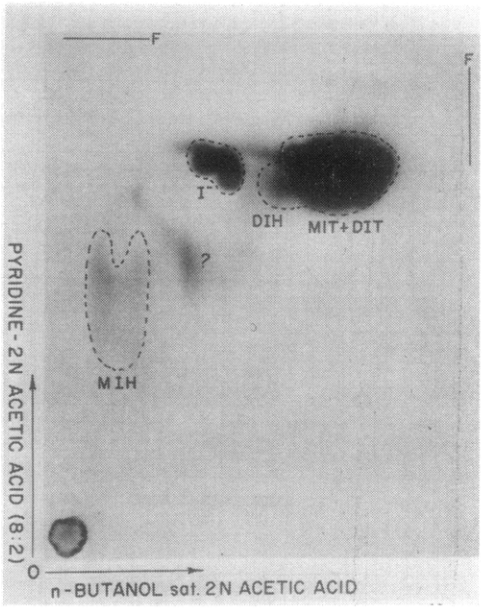


FIGURE 4: Autoradiogram of a two-dimensional chromatogram of lysozyme iodinated in 8 M urea, pH 8.50, +24°, with 12.0 moles of I_2/mole of protein, dialyzed and digested with pronase. 0 = origin; F = fronts of the solvents. First solvent: pyridine–2 N acetic acid (8:2); second solvent: 1-butanol saturated with 2 N acetic acid. Carriers are indicated by the dashed lines.

TABLE II: Disposition of Iodine after Iodination of Lysozyme at +24°.

Conditions of Iodination	Moles of I_2 per Mole Lysozyme	Bound[a]	Unreacted[b]	Lost[c]
H_2O	2	2.0	0	0
24°	4	3.7	0	0.3
pH 8.5	6	4.2	0	1.8
	8	4.7	0.8	2.5[d]
	10	4.8	1.8	3.4
	12	5.0	3.0	4.0
	14	5.0	4.7	4.3
8 M Urea	2	2.0	0	0
24°	4	3.7	0	0.3
pH 8.5	6	5.0	0	1.0
	8	5.7	0	2.3[d]
	10	6.3	0	3.7
	12	6.8	0	5.2
	14	7.0	0	7.0

[a] As calculated from the specific activity of the triiodide solution. [b] As calculated by titration with $Na_2S_2O_3$. [c] Obtained by difference; I_2 lost = total I_2 added − (I_2 bound + I_2 unreacted). [d] First appearance of a faint yellow color remaining after addition of reducing agent.

there was no effect and such concentrations also did not prevent the precipitation seen with iodination in H_2O.

In H_2O addition of more than 4–6 moles of I_2/mole of lysozyme led to precipitation of part of the protein. Such precipitation was previously noted by Fraenkel-Conrat (1950). The fraction precipitated was proportional to the amount of I_2 added and increased from 0.25 to ~0.70 as I_2 was increased from 8.0 to 14.0 moles/mole of protein at 24°. This precipitation commenced at the same [I_2]/[lysozyme] ratio at both 0° and 24° but was less extensive at the lower temperature, ranging from <0.10 to ~0.50 over the same spread of molar ratios. Although the precipitate was not studied in detail, it was found that when the degree of iodination was relatively low, the precipitate resembled the protein of the supernatant solution. With more extensive iodination, the precipitate was less iodinated than the fraction remaining in solution. In 8 M urea no precipitation occurred. However, on dialysis of lysozyme iodinated in urea, approximately the same fraction of the protein precipitated that would have been expected from iodination in H_2O under the same conditions.

Nature of the Iodinated Residues. The composition of lysozyme iodinated under various conditions has been examined by chromatographic analysis. A typical example of the results obtained is given in Figure 3. Lysozyme preparations chromatographed after iodination, reduction, and extensive dialysis showed only material at the origin (Figure 3A). Note that iodide is not present. Lysozyme iodinated with an [I_2]/[lysozyme] ratio of 0.9 contained mostly MIT (66%), the remainder being DIT. Digestion was complete in 5 hr and no deiodination occurred (no I^- spot) (Figure 3B). When the [I_2]/[lysozyme] ratio was increased to 4.8 (Figure 3C), DIT became the major iodinated amino acid and MIT was virtually absent. This is presumably a function of the greater fraction of the MIT present as the phenolate ion at this pH since the rate of iodination corrected for ionization is greater for tyrosine (Mayberry *et al.*, 1964).

Two additional compounds were present whose positions correspond to iodide and monoiodohistidine. That the compound with low R_F is, in fact, monoiodohistidine and not small peptides of MIT or DIT was shown in other solvents where MIH has greater mobility (Figure 4). MIH was found only when iodination was fairly extensive (>2 moles of I_2/mole of lysozyme). Since iodide was also found only under these conditions, we have assumed that it derived primarily from the deiodination of iodohistidines. 2,5-Diiodohistidine was seen only after extensive iodination but it did not increase as a direct function of the amount of I_2 added.

The difference between the total organic iodine and that recovered as MIT and DIT increases until a plateau is reached when the [I_2]/[lysozyme] molar ratio becomes

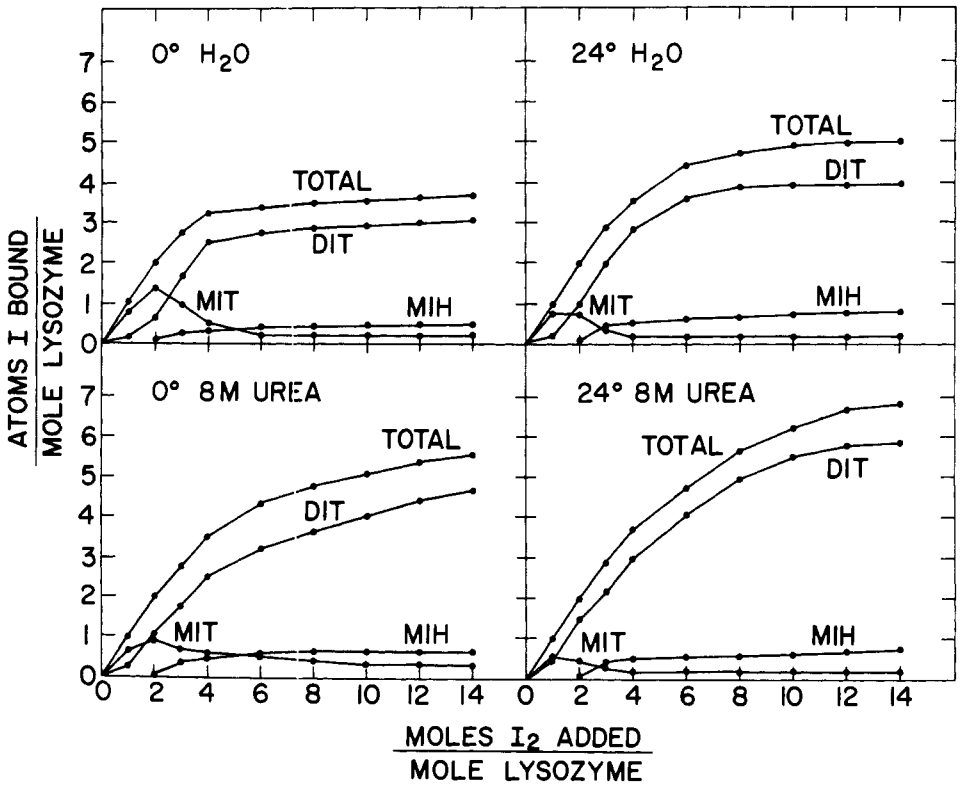


FIGURE 5: Organic iodine formation as a function of the molar ratio [I_2]/[lysozyme]. Iodine was added over a period of 60–90 min. Curves labeled H_2O are in 0.2 M Tris buffer pH 8.50. Curves labeled 8 M urea contain the same buffer.

about 5 and 7 for the lysozyme iodinated in water and in 8 M urea, respectively (Figure 5). This difference is entirely accounted for by the iodohistidines and iodide (the latter being presumably derived from iodohistidines as discussed above). Thus, for example, in water at 0° with 14 moles of iodine/mole of lysozyme, 1.65 moles of DIT, 0.3 mole of MIT, and 0.40 mole of MIH were found. Under the same conditions, but in urea, DIT = 2.25 moles/mole of lysozyme, MIT = 0.40, and MIH = 0.60. At 24° the corresponding values are DIT = 1.95, MIT = 0.10, MIH = 0.45, DIH = 0.20 moles/mole of lysozyme in H_2O, whereas tyrosine iodination is essentially complete in urea: the iodinated amino acids per mole of protein are DIT = 2.95, MIT = 0.10, MIH = 0.45, and DIH = 0.20. Thyroxine was not found under these experimental conditions.

Discussion

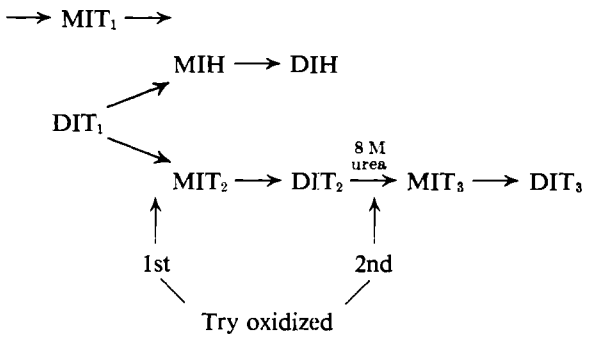
In the presence of excess iodine, two of the three tyrosyl residues of lysozyme were rapidly iodinated in H_2O, whereas the third or abnormal tyrosine was not and required the presence of 8 M urea to become iodinated. These reactivities resemble those toward cyanuric fluoride $(CNF)_3$ found by Kurihara *et al.* (1963). Of the two normal tyrosyl residues one may be fully iodinated before the other since the first two iodine atoms bound are recovered primarily as DIT. It is possible, of course, under these conditions, that two different tyrosyl loci each contribute a fraction of a DIT residue. This seems unlikely, however, since with an [I_2]/[lysozyme] ratio of 0.9, the predominant iodoamino acid is MIT. In addition, the speed of iodination is greater in urea, especially of the second tyrosine, suggesting that it may differ from the first (Figure 1).

Monoiodohistidine began to appear only when more than 2 moles of I_2/mole of lysozyme were used. Although strongly suggested (Fraenkel-Conrat, 1950), unequivocal evidence for iodohistidine formation in lysozyme has not been previously supplied. If we make the reasonable assumption that the iodide found after hydrolysis with pronase derives from MIH (or DIH), MIH accounted rather well for the difference between total organic iodine formed and that accounted for by MIT and DIT. Theoretically, 25% of the total organic iodine could be present in the single histidyl residue of lysozyme. In fact, only about half this amount of histidine iodine was recovered. Since only traces of DIH were seen on chromatograms, it seems likely that, unlike the tyrosines, monoiodination is the predominant event in the histidyl residue of lysozyme. We have obtained no evidence for or against the postulated *N*-iodoimidazole intermediates (Bauer and Strauss, 1936; Brunings, 1947) which can be deiodinated by reducing agents before rearranging to more stable C–I bonds. There was no significant difference in the yield of organically bound iodine whether thiosulfate or sulfite were used to stop the reaction, or when relative amounts

of I_2 were used that were completely consumed in the reaction and without subsequent addition of reducing agents. Koshland *et al.* (1963) were also unable to find evidence for N–I intermediates in the iodination of arsonic antibody with ICl.

The reason that iodohistidines are not described more often in iodinated proteins may be due, in part, to their extreme lability (Fraenkel-Conrat, 1950; Koshland *et al.*, 1963). It is likely, however, that they are formed; *e.g.*, Springell (1961) found up to 12 g-atoms of organic iodine bound to iodinated insulin. Since only four tyrosyl groups are present, the difference is well accounted for by the two histidyl residues.

If the three tyrosines are arbitrarily numbered 1, 2, and 3, the sequence of iodinations as a function of increasing [I_2]/[lysozyme] ratio may be viewed as follows



Two tryptophanyl residues are oxidized in the fully iodinated lysozyme. The importance of this is discussed in the following paper (Wolff and Covelli, 1966).

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