PROTEIN–IODINE INTERACTION

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I. INTRODUCTION

Iodination constitutes a unique means of conferring biological activity on inert proteins. Investigations on the iodination of proteins date back to the latter part of the nineteenth century, following the discovery of iodine in organic combination (15), and were encouraged by the finding that the normal thyroid gland itself contains organic iodine (102) as an essential constituent. These investigations were the result of the widely held belief that it would be possible to imitate the activity of the naturally occurring protein thyroglobulin by introducing iodine into other proteins. More recently, direct iodination of proteins has been investigated for a variety of reasons: to obtain thyroactive iodinated proteins and to study their mode of action; to determine the active centers in enzymes and hormones; to suitably alter proteins by introducing heavy atoms in known positions for crystallographic studies on the structure of proteins; to determine side chains involved in interactions with other reagents and responsible for characteristic titration phenomena; and to modify the antigenicity of proteins.

The term protein–iodine interaction is used herein to denote reactions that

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occur when proteins are treated with iodine. The chemical aspects are stressed in terms of the amino acids responsible. Brief mention is made, however, of the fixation of iodine by the thyroid gland and the mode of conversion of 3,5-diiodotyrosine to thyroxine in iodoproteins. Loosely bound iodine complexes of proteins in the circulating plasma will not be included. The literature on the subject has been surveyed up to September, 1955. Direct reference is made only to a select number of papers, and the review constitutes a factual appraisal of our knowledge on this subject. For purely biochemical aspects of iodoproteins and certain limited discussions of reactions between proteins and iodine, the reader's attention is drawn to several reviews (3, 52, 61, 64, 83, 124, 157, 173).

II. TYPES OF REACTIONS BETWEEN IODINE AND PROTEINS

The action of iodine on proteins, or on their constituent amino acids, in aqueous or non-aqueous solutions can theoretically be either of one type or a combination of three types: (*a*) addition, (*b*) substitution, and (*c*) oxidation. No clear demonstration of simple addition of iodine to a protein has been made, although work with methionine (105) indicates the possibility of adding iodine to the thioether linkage to form a perhalide. *N*-Acylated derivatives of methionine would, however, give rise to only unstable perhalides.

In principle, the mode of reaction with iodine may be evaluated in the following ways: (*1*) simple addition would involve loss of free halogen from solution without concomitant formation of inorganic iodide; (*2*) in a substitution reaction the amount of iodine organically bound would equal the inorganic iodide formed in solution

$$RH + I_2 = RI + HI$$

(*3*) when the reaction is oxidative no iodine would be organically bound, the iodide ion formed equalling the loss of free iodine. The latter two types are widely encountered with amino acids or proteins, depending on the conditions of reaction. In these reactions estimations of the loss in free iodine, of the organically bound iodine, and of the iodide ion newly formed in the same system would enable precise calculation of the extent of each type.

III. ACTION OF IODINE ON THE SULFUR-CONTAINING AMINO ACIDS

A. The sulfhydryl group: cysteine

Iodine in acid media has a high oxidation potential, which decreases with increase in pH. In dilute solutions of high iodide content fairly specific oxidation of —SH groups will occur. Thus, such groups in native ovalbumin may be oxidized completely at 0°C. and pH 3–5 by stoichiometric amounts of iodine in 1 *N* potassium iodide (5, 6, 7, 8, 183). At higher temperatures the oxidation proceeds beyond the disulfide stage. On the other hand, the —SH groups of tobacco mosaic virus react with iodine at neutrality only in solutions of low iodide concentration and in the presence of excess iodine,—2.5 times the stoichiometric amount (9). The virus retains its activity under these conditions.

Recent work on tobacco mosaic virus (51) confirms that about two atoms of iodine at pH 6–8 abolish the sulfhydryl groups. Analyses revealed that half of the iodine added was, however, bound by the nucleoprotein, the substitution reaction being represented as follows:

$$RSH + I_2 \rightarrow RSI + H^+ + I^-$$

The sulfenyl iodide so formed was stable, and denaturation of the protein restored the characteristic lability and reactivity of the grouping. No other —SH proteins yielded such a derivative, although it might be involved as a transient intermediate. As a reagent for sulfhydryl groups, iodine has been widely used in the study of —SH enzymes (17, 18, 76, 77, 122, 185).

This first step in the oxidation of cysteine to cystine, in the free state or in protein combination, may be represented as:

$$RSH + I_2 = RSI + HI \quad (1)$$

$$RSI + RSH = RSSR + HI \quad (2)$$

and the overall reaction as:

$$2RSH + I_2 = RSSR + 2HI$$

$R = -CH_2CH(NH_2)COOH$.

The disulfide bond, in turn, is also capable of being oxidized.

B. The disulfide linkage: cystine

Cysteic acid, which is the main product of the oxidation of cystine by iodine, is easily prepared (181). Early studies on the iodination of proteins (23) also recognized the possibility that cystine in proteins may be oxidized to cysteic acid. This was deduced from the negative tests for reduced sulfur and from the formation of iodoform and large amounts of hydriodic acid during iodination. However, no direct evidence for the final oxidation product was available. The identification of cysteic acid in hydrolysates of iodinated casein has recently been achieved (152). Furthermore, analyses for cystine by the Folin and Marenzi method, using the uric acid reagent, indicated the presence of products having higher chromogenic value than cystine, although the nitroprusside test indicated complete absence of the amino acid in the iodinated casein.

The reaction between cystine and iodine may be represented as follows:

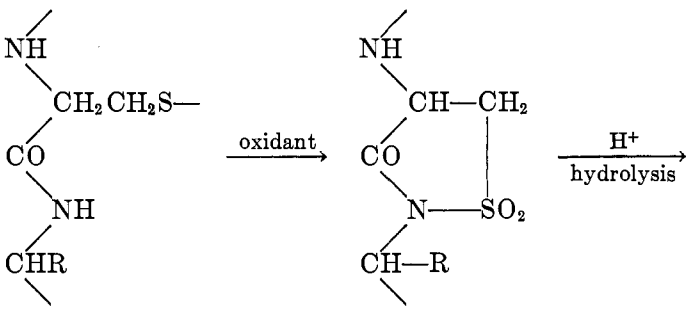
$$RSSR + 2I_2 + 3H_2O \rightarrow 2RSO_3H + 4HI$$

However, the information available on the oxidation of disulfides by other oxidizing agents suggests the possibility of intermediate steps in the reaction.

Thus, cystine is oxidized by perbenzoic acid to a thiosulfonate,[2] which is readily converted to the corresponding sulfonic and sulfinic acids (106).

$$RSSR \xrightarrow{C_6H_5CO_3H} RSO_2SR \xrightarrow{\text{oxidation}} RSO_3H + RSO_2H$$

Hydrogen peroxide gives cysteic acid as the main reaction product, with some evidence for an intermediate sulfoxide or thiosulfonate (71). Bond fission was assumed to occur only after one of the sulfur atoms was converted to the thiosulfonate. Studies on the oxidation of wool by bromine (41) and by hydrogen peroxide (40) indicate the possible occurrence of the sulfinic acid and the sulfoxide as intermediates. Of further interest is the report (4) that oxidation of wool by peracetic acid results in the possible formation of a cyclic sulfocarboxylic acid imide, which on acid hydrolysis would yield cysteic acid:



$HO_3SCH_2CH(NH_2)COOH$

It is thus possible that the oxidation of the disulfide bond in free and protein-bound cystine by iodine could easily involve intermediates encountered with other oxidizing agents, although direct evidence is lacking.

C. The thioether linkage: methionine

Organic sulfides or thioethers are characterized by their reaction with halogens to form perhalides and addition compounds of the type

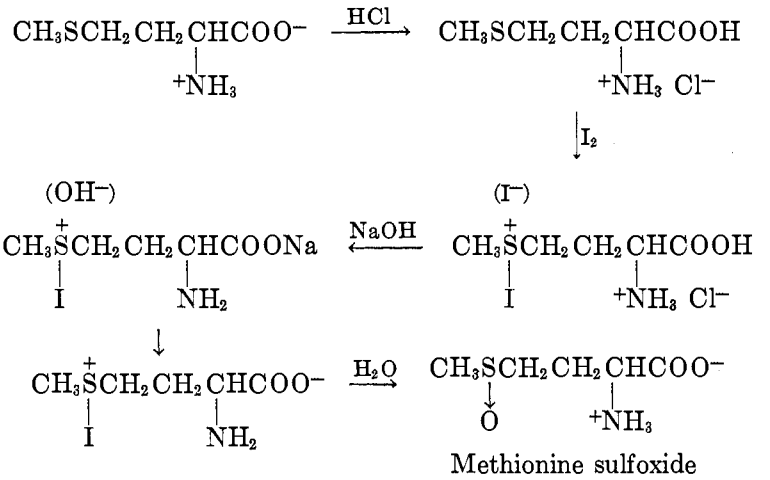
$$R_2S + X_2 \rightarrow [R_2SX_2] \rightleftharpoons [R_2SX]^+X^-$$

which are more or less readily hydrolyzed to sulfoxides, according to the following equation:

$$[R_2SX]^+X^- + H_2O \rightarrow R_2SO + 2HX$$

That methionine, by virtue of its thioether structure, is also capable of these reactions has been demonstrated (105). The methionine periodide formed with iodine is colorless and soluble in water. Its rate of formation increases and rate of hydrolysis decreases with increasing acidity. In solution it has been suggested that an iodosulfonium hydroxide is formed. At the present time it seems that the following formulas might represent the transformations.

[2] The authors (106) and other earlier workers assigned a disulfoxide structure to the intermediate, but recent work has shown that all such compounds are actually thiosulfonates.



Methionine sulfoxide

Cysteine (51) and, to a lesser extent, cystine seem to be capable of forming some type of periodide.

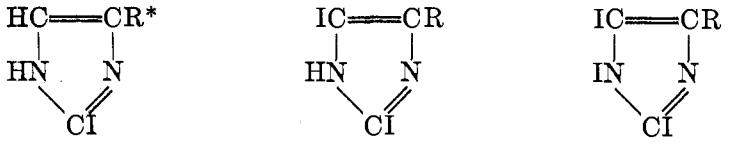
Practically nothing, however, is known of the influence of iodine on the thioether linkage in proteins. Since periodides of *N*-acylated methionine are unstable (105), methionine periodide cannot, perhaps, be a constituent of iodinated proteins. But it is probable that the hydrolysis product of methionine periodide —namely, methionine sulfoxide (see above)—occurs bound in iodinated proteins. Iodination of casein in 50 per cent acetic acid at 5°C. and 37°C. effects a decrease in the methionine content of the products (152), as measured by the nitroprusside reaction (83, 89). Chromatography of hydrolysates of the iodinated casein indicates the presence of appreciable amounts of methionine sulfoxide (151).

IV. ACTION OF IODINE ON HYDROXYAMINO ACIDS: SERINE AND THREONINE

An appreciable drop in the hydroxyamino acid content of casein was observed when iodination was effected in 50 per cent acetic acid or in 5 per cent ammonium hydroxide at 37°C. (152, 153), the destruction being less at 5°C. At neutrality, in 1 per cent sodium bicarbonate, or in 0.5 *N* sulfuric acid iodine causes extensive destruction of free serine at 70–100°C. and moderate destruction at 37°C. The destruction is least in neutral media. Neither the acid nor the bicarbonate alone effects any appreciable destruction under these conditions. With threonine the destruction is not marked; with 5-hydroxylysine it is inappreciable. It was considered, therefore, that a long carbon chain attached to the carbon carrying the hydroxyl group makes the compound less susceptible to the action of iodine. Paper chromatographic examination of the reaction mixture obtained with serine shows the absence of the amino acid (ninhydrin test) and the presence of a carbonyl compound. The products of oxidation have not been studied further and would appear to merit examination.

V. ACTION OF IODINE ON IMIDAZOLE GROUPS: HISTIDINE

Early work (143) recognized that iodine reacts with imidazole and benzoylhistidine. Substitution in the latter compound occurs both on the imino group and on the ring carbon. The iodine of the =N—I group is easily removable by bisulfite, a fact which, together with other work (144, 145), indicated that imidazole can substitute one atom of iodine on the imino group and three atoms on the ring carbons, under suitable conditions. Since one of the ring carbons in histidine carries a side chain, the substitution products will be:



2-Iodohistidine 2,5-Diiodohistidine 1,2,5-Triiodohistidine

* R = $-CH_2CH(NH_2)COOH$.

The amount of iodine that will actually combine with histidine was found to depend in large measure on the reaction conditions, the uptake of iodine being more rapid in more alkaline solution. The iodine taken up by carbon was found to be in firm combination. Reaction between protein-bound histidine and iodine was first demonstrated to occur in the case of sturine, which bound an amount of iodine equivalent to its histidine content.

Although Pauly was unable to iodinate free histidine directly, reaction between the two has been found possible, and 2-iodo- and 2,5-diiodohistidine have been synthesized (31). Both compounds give a weak ninhydrin test and a moderately strong reaction in Pauly's diazo test.

Products obtained by the prolonged iodination of casein in a bicarbonate medium were found to have high iodine content (23), which was lowered considerably by treatment with bisulfite. Since the content of residual iodine corresponded to that of products obtained by short-term iodination, the iodine thus removed was presumed to have been in loose combination with the imidazole groups in the protein. Iodination of globin, which is rich in histidine, indicated fixation of iodine by these residues, although, oddly enough, such residues in egg albumin, serum albumin, and globulin did not bind iodine (12–14). Iodinated insulin also showed no evidence of containing iodohistidine (111). Monoiodohistidine has been reported to be present in iodinated globin and in thyroglobulin (168). In the case of lysozyme it has been found that, under conditions in which half the phenolic groups of tyrosine remained unreactive toward iodine, the single residue of histidine present participated fully (50), causing a reversible inactivation.

The rate of iodination of histidine has been found to be 30–100 times slower than that of tyrosine (108), indicating that histidine in proteins would react if iodine were present in excess and the duration of treatment prolonged. However, the iodination of free histidine requires more vigorous conditions than those for histidine anhydride (31), so that imidazole groups in proteins may react at

rates vastly different from those observed with histidine. Chromatographic examination of products in reaction mixtures of varying proportions of iodine and histidine (166) indicated the simultaneous presence of both the monoiodo and the diiodo derivatives. This observation was considered inconsistent with the conclusion (108) that the formation of the monoiodo derivative would be the rate-determining step in the formation of diiodohistidine. The possibility that excess iodine and prolonged treatment may rupture the imidazole ring, especially in alkaline media, was also recognized (166).

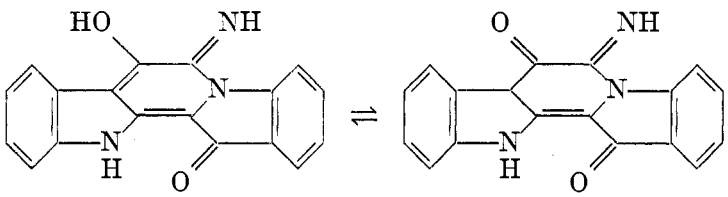
VI. ACTION OF IODINE ON INDOLE GROUPS: TRYPTOPHAN

Early reports indicated the possibility that tryptophan can form iodinated derivatives. The isolation of monoiodotryptophan (101, 132) and diiodotryptophan (132) has been reported. Reference to the preparation of a diiodotryptophan and its lack of physiological activity is also to be found (118). Others (2, 131) have considered the possibility of the formation of such derivatives when proteins are treated with iodine under certain conditions.

Iodine does react vigorously with both free and combined tryptophan, but in spite of the earlier researches the nature of the products still is not clear. Iodinated proteins give negative Adamkiewicz and Ehrlich tests, and it has been suggested that the α-CH of the pyrrole ring in tryptophan in proteins is oxidized by iodine (13). Unsuccessful efforts to isolate iodinated derivatives also have been reported (143). Recent work (170, 171) shows that the progressive diminution of tryptophan during iodination, as estimated by the Lugg method, may be explained by the formation of iodinated derivatives or by oxidation. But the nonregeneration of the original tryptophan by dehalogenation with alkali–stannous chloride, and the fact that free tryptophan in borate or bicarbonate buffer (pH 8.4) reacts with seven to eight atoms of iodine per molecule (reducing the iodine to iodide), led to the conclusion that oxidation was the more probable. These observations find support in other investigations (153) where the reaction was found to be solely oxidative at pH 8.1. The Millon test and the Ehrlich aldehyde test were negative after reaction with one molecule of iodine, despite the fact that the reaction product retained the ability to reduce six more atoms of iodine. It was considered possible that the initial oxidation converts the α-CH of the pyrrole ring to —C(OH), and that in later stages the side chain and the pyrrole cyclic structure become ruptured. Although the products of some of these reactions were isolated (151), no detailed study has been made. At a slightly lower hydrogen-ion concentration, pH 7.0, acetyltryptophan and gramicidin (40 per cent tryptophan) rapidly decolorize iodine (52). This indicates that indole groups in proteins react in similar fashion. However, although free tryptophan reacts with its equivalent of iodine at even a much lower pH, 3.2–3.4 (5, 111), the tryptophan units in chymotrypsinogen, pepsin, and lactogenic hormone remain unreactive under the same conditions.

Tryptophan can react with iodine also to yield pigments. Free tryptophan gives a color reaction with iodine in its isoelectric region, pH range 4–6 (47, 48). Earlier workers (1, 133) had observed pigment formation only with chlorine or

bromine. Pigment also develops outside the pH region 4–6 if a phosphate buffer is employed (153). The pigment formed at pH 8.1 with two atoms of iodine per molecule of tryptophan is reddish in color with an intense bluish fluorescence. With larger amounts of iodine, up to six atoms, the reddish pigment persists, but the fluorescence diminishes appreciably. With eight atoms of iodine, or more, a yellow product results which is not converted to the red pigment by addition of tryptophan as was the yellow product obtained by Neuberg (133). The red pigment absorbs strongly at 420 mμ and below 320 mμ. The material is a mixture of several pigments, probably arising by initial oxidation at the α- and β-positions of the pyrrole ring, and condensation of the ring with the substituted indoles present in the system to yield compounds of the Hopkins–Cole type. The pigment formed at pH 5.6 with two atoms of iodine in phosphate buffers has been fractionated into at least three components, one of which has been identified as tryptochrome on the basis of its absorption characteristics (152). This compound has been prepared by treating free tryptophan in glacial acetic acid with potassium iodate and is provisionally assigned the following structure (48):



$[C_{18}H_{11}O_2N_3]$

The ability of tryptophan to reduce iodine to iodide has been used for estimating this amino acid in proteins, after partial fractionation of hydrolysates through the mercury complex according to the method of Lugg, and found to yield satisfactory results (152).

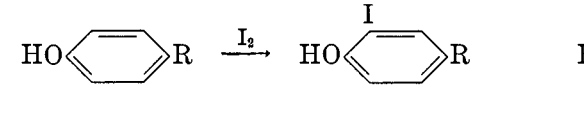
VII. ACTION OF IODINE ON PHENOLIC GROUPS: TYROSINE

A. Naturally occurring iodo derivatives of tyrosine

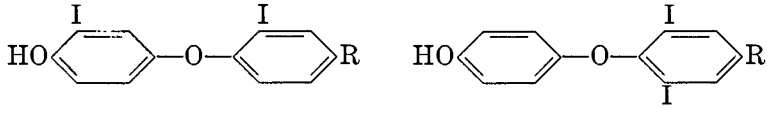
One of the main products of the iodination of tyrosine, 3,5-diiodotyrosine, was first isolated from several iodinated proteins (46, 136–141); its presence in thyroglobulin was discovered later (70). Soon another crystalline, physiologically active material, thyroxine, was isolated from the thyroid gland in small yield (95, 96); its structure was incorrectly considered to be trihydrotriiodoöxindolepropionic acid. The isolation of this compound in better yield was achieved (63) and its structure definitely established as β-[3,5-diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)phenyl]-α-aminopropionic acid. The synthesis of the racemic compound was later achieved (66) and also that of *l*-thyroxine, starting from *l*-tyrosine (29, 35). In addition to thyroxine, 3-iodotyrosine was isolated (116) from iodinated casein, and the presence of this amino acid in iodopepsin (80), in thyroglobulin (49, 58, 184), and in gorgonin (54) was soon established. 3-Iodo-(*l*)-tyrosine has been synthesized (68) from *l*-tyrosine through the intermediate 3-nitro-(*l*)-tyrosine and 3-amino-(*l*)-tyrosine.

The occurrence of another derivative of tyrosine, 3,5-diiodothyronine, and evidence for the possible presence of 3,5,3′-triiodothyronine in iodinated casein were also reported (84). Diiodothyronine was soon found also in thyroglobulin (172), and the compound was shown to be readily convertible to the triiodo derivative by partial iodination (169). The occurrence of triiodothyronine in the thyroid gland and in plasma was later established (59, 177). Subsequently, the properties of this compound have been investigated (60). In a very recent report the presence of two other iodinated amino acids in the thyroid gland is indicated (178). The compounds are 3,3′-diiodothyronine and 3,3′,5′-triiodothyronine.

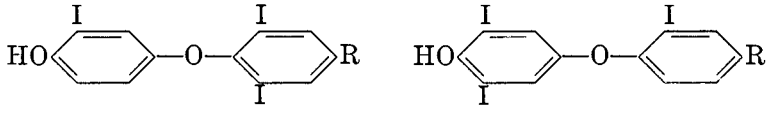
The sequence of products resulting from the action of iodine on tyrosine is as follows:



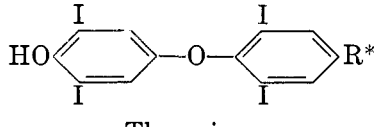
Tyrosine 3-Iodotyrosine 3,5-Diiodotyrosine



3,3′-Diiodothyronine 3,5-Diodothyronine



3,3′,5-Triiodothyronine 3,3′,5′-Triiodothyronine



Thyroxine

* R = $-CH_2CH(NH_2)COOH$.

The compounds having the diphenyl ether linkage are not strictly products resulting from the direct action of iodine on tyrosine but result from oxidative coupling between tyrosine and partially or fully substituted derivatives of tyrosine. The coupling reactions are possibly catalyzed by iodine (94) or hypoiodite (69).

B. Kinetic studies on the formation of 3,5-diiodotyrosine from tyrosine and iodine

In the process leading to the formation of diiodotyrosine from tyrosine and iodine, the formation of monoiodotyrosine has been suggested as the rate-determining step, the overall reaction being catalyzed by acetate and phosphate (107, 109). The rate was shown to be inversely proportional to the hydrogen-

ion concentration and the square of the iodide concentration, and directly proportional to the buffer concentration. Of at least four combinations of reactive species—namely: (*1*) iodine and the free phenol, (*2*) iodine and the phenoxide, (*3*) hypoiodous acid and the phenol, and (*4*) hypoiodous acid and the phenoxide—the last was considered the most important in the formation of iodinated tyrosine in neutral or slightly alkaline solution. Evidence from other sources questions the validity of the claim that the formation of monoiodotyrosine is the rate-determining step in the conversion of tyrosine to diiodotyrosine. For example, it has been pointed out that monoiodotyrosine is a stable intermediate in this process (167). The isolation of monoiodotyrosine from several iodinated proteins (50, 80, 116) is further evidence against the claim. If the introduction of the first iodine atom were the rate-controlling step, little monoiodotyrosine would be detected in reaction mixtures at any particular instant during the course of reaction. The discrepancies pointed out suggest a reëxamination of the kinetics and mechanism of this reaction.

C. Effect of introduction of iodine on the properties of tyrosine

When iodine is introduced into tyrosine (free or protein-bound), modifications in the ultraviolet spectrum take place (55, 119, 159) as well as changes in the titration curve (67, 85, 134). In the case of iodinated globin the expected displacement of the pK value for the phenolic hydroxyl group (pK = 10.0) toward the acid region does not occur (39).

D. Iodine as a group-specific reagent for tyrosine in the study of proteins

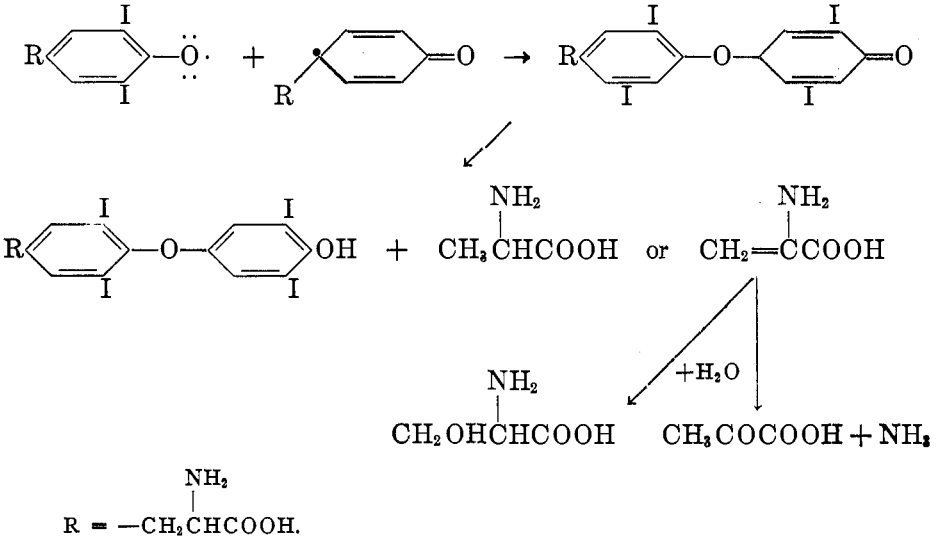
A variety of proteins have been iodinated to convert the tyrosyl residues to diiodotyrosyl, and thereby to study the role of the phenolic groups in biological activity. Iodinated insulin with all tyrosine residues iodinated was found to have only 10–15 per cent of the original activity (67). Catalytic hydrogenation resulted in removal of 70 per cent of the iodine and in the restoration of activity to the extent of 30–50 per cent. The inactivation of insulin has been, however, considered also on the basis of disruption of the dithiol linkage by the oxidative action of iodine in alkaline solutions (93). Iodination studies on pepsin suggest that the phenolic groups of tyrosine are essential for biological activity (79). Examination of the biological activity of iodinated derivatives of lactogenic hormone (111), growth hormone (113), tobacco mosaic virus (9), adrenocorticotropic hormone (112), and chorionic gonadotropin (19) have shown that the phenolic groups of tyrosine may be essential for biological activity in these proteins. But the acceptance of iodine as a group-specific reagent would seem to need more study, because of the variety and complexity of concomitant side reactions under even the most favorable conditions chosen for the iodination of the tyrosyl groups.

Further, the reactivity of the phenolic groups in proteins toward iodine varies. Thus, rate measurements on pepsin and serum albumin (110) indicate that not all tyrosine radicals in a given protein are iodinated under mild conditions nor all at the same rate, and that "availability" may be a factor. The rate of

reaction was found to be a function of the degree of denaturation. At pH 5.7 in acetate buffer, 50 per cent of the groups were unavailable in serum albumin, and even after urea-heat denaturation, 33 per cent of the groups were still unreactive. In native pepsin only 88 per cent of the groups were found to react. Under comparable conditions, nearly three out of nine tyrosyl groups in the molecule of egg albumin were unavailable for iodination (154). Urea-heat denaturation did not alter the reactivity of these groups, but the use of a phosphate buffer of the same pH, instead of acetate, resulted in activation and availability of all the groups for full iodination. These observations on the availability of tyrosine groups in proteins for iodination point to difficulties to be overcome in using iodine as a satisfactory group-specific reagent.

E. The problem of thyroxine formation in in vivo and in vitro systems

The simultaneous presence of diiodotyrosine and thyroxine in iodoproteins suggests that thyroxine is formed from diiodotyrosine. The overall reaction is thought to involve the iodination of tyrosine, followed by the oxidative coupling of two molecules of diiodotyrosine and the elimination of a side chain (65). This type of reaction is analogous to the oxidation of *p*-cresol by potassium ferricyanide (150) and has strong experimental support in observations made with diiodotyrosine (94). The sequence of reactions may be represented as below:



The presence of serine (135), pyruvic acid, and ammonia in the reaction mixtures has been detected. Iodine (94), or hypoiodite (69), is considered the actual oxidizing agent. Thus hydrogen peroxide and oxygen have an augmenting effect on thyroxine formation. The catalytic effect of Mn_3O_4 and agitation in the presence of air on the reaction have also been reported (160). It has been found that antithyroid substances, which can reduce iodine, have an inhibitory effect on the coupling (147).

The identification of alanine in the reaction mixture indicated that the coupling reaction could be dismutative, rather than oxidative (148), but this product could be the result of other secondary reactions involving pyruvic acid formed in oxidative coupling.

While the optimum pH for thyroxine formation from free diiodotyrosine is approximately 10, in the case of diiodotyrosyl peptides it is closer to the pH of tissues involved in the biosynthesis of thyroxine (148). The elaboration of thyroxine in the thyroid gland from tyrosine in thyroglobulin occurs via monoiodotyrosine and diiodotyrosine (34, 124). The possible intervention by cytochrome oxidase (130), peroxidase (43, 163), xanthine oxidase (98), or manganese (156) also has been pointed out. The elaboration of diiodothyronine and triiodothyronine possibly occurs through oxidative coupling of diiodotyrosine with tyrosine or monoiodotyrosine (84, 177), although dehalogenation mechanisms have also been considered (61).

VIII. METHODS OF IODINATION OF PROTEINS

Iodine may be added to proteins in a variety of forms. Many early investigations have been concerned primarily with obtaining products of constant composition corresponding to definite degrees of iodination, often with the formation of thyroxine in view. As a result, in such studies the choice of an iodinating agent which would, for example, induce a minimum of denaturation was insufficiently considered. At first, the iodine for reaction was liberated in acid media (21, 25, 26, 86, 103, 104) by the well-known reaction

$$5KI + KIO_3 + 3H_2SO_4 \rightarrow 3I_2 + 3K_2SO_4 + 3H_2O$$

and breakdown of the protein by liberated hydrogen iodide prevented by the addition of magnesium carbonate and sodium bicarbonate (26). Iodinated casein was also prepared by heating the protein and pulverized iodine together at 100°C. (115), the products obtained being of unusually high iodine content (17.5 per cent), part of such iodine being held in loose combination and readily removed by alkaline bisulfite.

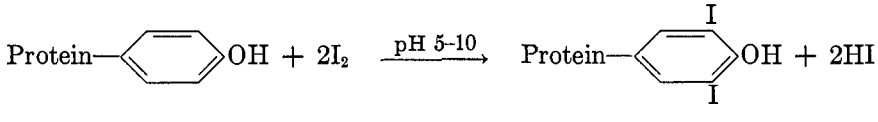
In more recent investigations effective iodination of proteins has been achieved in aqueous solution through the use of pulverized iodine, solutions of iodine in alcohol (with little iodine present), solutions of iodine in aqueous potassium iodide, solutions of iodine in potassium iodide plus strong ammonium hydroxide (involving perhaps the unstable intermediate nitrogen triiodide), and by the use of hypoiodite. Buffers and the use of sodium bicarbonate media have been favored to remove hydrogen iodide formed during reaction. The iodination of proteins has also been carried out in 50 per cent acetic acid. The amino acid residues participating in the reactions are cysteine, cystine, methionine, histidine, tyrosine, tryptophan, and the hydroxyamino acids,—each of which has been considered in some detail in this review.

Although it was recognized early that the iodine content of a preparation and the groups concerned vary according to the conditions employed, there have been few systematic evaluations of the relative effectiveness of the various iodi-

nating agents. In a recent study a comparison was made between the use of 50 per cent acetic acid, 5 per cent ammonia, and 1 per cent sodium bicarbonate as media for the iodination of casein (152), with reference to the amount of iodine introduced into casein. The iodine was added in all cases as pulverized iodine in the proportion of 2, 4, and 8 g. of iodine per 20 g. of protein in various experiments. The amount of iodine substituted (7.0 per cent) with 8 g. of iodine in 5 per cent ammonia at 5° and 37°C. and in 1 per cent sodium bicarbonate at 37°C. seemed to correspond to full substitution of all tyrosine radicals. With 50 per cent acetic acid the amount substituted under these conditions was low (a maximum of 4.55–4.77 per cent), temperature having no appreciable effect. With the ammonia and sodium bicarbonate an increase in temperature markedly affected the amount of iodine substituted. The increased substitution in sodium bicarbonate at 70°C. was probably due to incorporation into groups such as imidazole. The highest substitution was achieved in bicarbonate at 70°C., using 8 g. of iodine per 20 g. of protein, indicating that these conditions are most suitable for introducing large amounts of iodine into the protein.

The suggestion that the highest incorporations of iodine into protein would be obtained in ammonia solution (188) may not be generally valid, and the destruction of amino acids, like methionine and cystine, due to oxidation in an ammonia medium (152) does not accord with the claim (36) that the use of the ammonia medium, while favoring full iodination of tyrosine, would eliminate the oxidation by hypoiodite in dilute alkaline solutions. This has been also questioned elsewhere (83) on the basis of the finding (37) that hypoiodite is an extremely effective iodinating agent for phenols and that even the iodinating action of nitrogen triiodide, present transiently in solutions of iodine in ammonia, is due to the hypoiodite which results from its action with water (179). At least with casein, while complete substitution of tyrosine occurs in an ammoniacal medium, more incorporation of iodine into the protein is possible in a bicarbonate medium (152).

The better-known substitution reactions of iodine with proteins involve tyrosine or histidine. At low iodide concentrations and at neutral or alkaline pH, iodine preferentially reacts with phenolic groups, giving rise to fully substituted diiodotyrosine residues:



However, minor oxidative side reactions may often precede the substitution reactions even at low iodine concentrations, and at high concentrations more than the tyrosine equivalent of iodine can be bound, as, for example, in the case of human serum albumin (90), where it was found that other substitution reactions occurred after approximately 70 per cent of the 3,5-positions in tyrosine had reacted. Such concomitant substitution as had occurred was appreciable and perhaps resulted from substitution in the imidazole ring.

Relatively small amounts of iodine in iodide solution at pH 5.0–6.0 or near

neutrality, at 0°C. or at room temperature, can yield iodinated products without the occurrence of appreciable denaturation. This is true of pepsin (80, 82), serum albumins (28, 90, 180), and thyroglobulin (126). Products obtained by the action of an excess of iodine and containing 6–14 per cent iodine are always denatured. The products obtained in these reactions vary with the mode and extent of iodination. A case in point is the iodopepsins, one of which with a high iodine content contained 80 per cent of the iodine as diiodotyrosine, and the other with a lower iodine content had 65 per cent of the iodine as monoiodotyrosine. The formation in these products of monoiodotyrosine and diiodotyrosine in relation to the amount of reagent allowed to react with the protein and other conditions of reaction have been studied (175, 176) with casein. Halogenation of thyroglobulin and zein in sodium bicarbonate solution or in ammoniacal solution also yielded the same results. Monoiodotyrosine was found to be formed in abundance in slightly iodinated proteins, while diiodotyrosine predominated in products richer in iodine.

The level of halogenation of proteins is dependent on the temperature, the concentration of the reagent, the pH of the medium, and the nature of the protein itself. Although earlier workers attempted to correlate the level of iodination with the composition and structure of the protein (13, 14, 23, 24, 114), in general it is difficult to state exactly the maximum amount of iodine which can be introduced into a protein in different forms of combination because of the several simultaneously occurring reactions with iodine and the near-impossibility of being able to control the occurrence of many of these reactions or their end-points.

Proteins, maximally treated with iodine under drastic conditions, undergo several side reactions resulting in negative tests for tryptophan due to oxidation, negative tests for reduced sulfur and formation of iodoform, destruction of many groups responsible for the biuret reaction, and formation of methyl iodide at the expense of methionine. The specific tests for non-ortho-substituted phenols disappear owing to substitution. And it is conceivable that the action of an excess of iodine on proteins may also involve extensive intramolecular alteration. Thus, there is considerable evidence for the destruction of tyrosine (6, 30, 39, 85, 146, 175). Rupture of peptide bonds adjacent to halogenated tyrosine residues would also appear possible from the observations made on the cleavage of oxytocin by bromine water (161).

During the iodination of proteins the formation of thyroxine from diiodotyrosine is a step quite distinct from simple iodination. The conditions for such oxidative coupling of diiodotyrosine residues to form thyroxine in iodinated proteins have been studied in considerable detail, and surveys of the literature on this subject are to be found elsewhere (157, 173).

IX. IODOPROTEINS AND SOME OF THEIR PROPERTIES

The only iodoproteins of any importance which occur naturally are thyroglobulin, occurring in the thyroid gland, gorgonin, and spongin, the latter two of which are present in the marine organisms Gorgonacea and sponges. The iodine in the proteins of Gorgonacea (128, 129) has been found to be in combina-

tion with tyrosine as monoiodotyrosine (54, 164) and as diiodotyrosine (42, 46, 78, 164, 165). Most of the iodine in sponges occurs as diiodotyrosine (42, 78, 141, 186). As proteins, gorgonins belong to the group pseudokeratins (20). The detailed study of the composition of the proteins of *Scirpearia flabellum* Johnson and *Elicella paraplexauroides* Stiasny has provided support for the claim of some zoologists that the two organisms are identical. A fuller discussion of the halogenated proteins of invertebrates is given in a recent review (173).

The most important of the naturally occurring iodoproteins is thyroglobulin. About 90 per cent of the iodine found in vertebrate thyroid glands is known to exist in combination with protein, and several investigators (16, 32, 57, 91, 92) have established the globulin character of this protein. The protein component was first separated, though in an impure state (136, 137), from saline extracts of the gland by half-saturation with ammonium sulfate followed by dialysis. Alcohol fractionation was subsequently used (11), and the method of isolation has been improved (33, 73) so as to eliminate the presence of nucleoproteins. More recent developments (44, 45) have avoided denaturation and made possible the elimination of lipoprotein and nucleoprotein impurities. The pure thyroglobulin obtained has been found to be electrophoretically homogeneous, with an average mobility of 4.54×10^{-5} cm.^{2}/volt. sec. in a phosphate buffer ($\Gamma/2 = 0.2$) of pH 7.65, retaining the homogeneous character between pH 6.2 and 7.7 (125). The sedimentation constant, S^0_{20}, of hog thyroglobulin is 19.4 S, and the molecular weight 650,000 (45, 74, 97). The isoelectric point is at pH 4.48 and the stability zone between pH 4.8 and pH 11.3 (74, 75). The protein displays heterogeneity (three fractions) when analyzed by the solubility method of Cohn and Northrop, but these fractions have the same nitrogen: iodine ratio. Under certain conditions some of these fractions behave like, or are changed into, one or another of the three. It has been observed also (117) that with a change in protein concentration and salt concentration, the thyroglobulin exhibits reversible dissociation and has the ability to form aggregates of uniform size. Normal thyroglobulins from various sources (125, 142, 187) have the same ratio (0.3–0.4) of thyroxine iodine to total iodine, although wide variations in total iodine in the thyroid gland and in thyroglobulin may be encountered, as for example materials isolated from Indian cattle, which are of uniformly high iodine content compared to similar materials isolated from European cattle (56, 151).

Among proteins whose iodinated derivatives have elicited early study are albumins (87), hemoglobin (27, 88), serum albumin, ovalbumin, serum globulin, thyroglobulin, nucleohistone, and gelatin. Others include zein (134, 176), insulin (67, 176), fibroin (127, 155), casein (22, 26, 86, 157), serum albumin (99, 131), serum globulin and globin (13), thyroglobulin (162), gliadin (173), vitellin (155), and several other proteins and peptones (176). Crystalline derivatives of serum albumins (28, 90, 180) and pepsin (80, 82) have been isolated. Thyroglobulin labelled with I^{131} has been isolated from the glands of animals to which labelled iodide has been administered (123, 174).

Iodinated proteins containing maximal amounts of thyroxine have also been

prepared and found to possess high biological activity (157, 173). Interesting analytical problems in the study of such products have been discussed in another review (149).

Proteins iodinated under mild conditions may be expected to retain their original structure without significant modification. Hence in the elucidation of microscopic protein structure, methods for chemical modification, like iodination, may hold promise. For example, useful information on the position of tyrosine residues (distant spacing) has been provided by x-ray diffraction studies (53). On the other hand, diffraction patterns of tyrosine and some of its iodinated derivatives have indicated some structural rearrangements (182), although thyroglobulin of varying iodine content retained the same pattern and no differences were found to exist in the patterns obtained with casein and iodinated casein.

Iodination of tyrosine residues increases the dissociation of the phenolic group a thousand fold, and in iodinated proteins the portion of the titration curve assigned to the phenolic group (pH 10) is usually displaced in the direction of increased acidity (38, 67, 79, 134) by nearly two pH units. Iodoglobin is an exception to this general observation (39).

In iodinated protein the ultraviolet absorption maximum arising from the phenolic groups is shifted toward higher wavelengths, owing to the iodination of positions ortho to the phenolic hydroxyl of tyrosine (120, 121, 158).

The dye-binding capacity of iodinated serum albumin (100) and the immunochemical properties of iodoproteins, in relation to problems in antigen–antibody relationship (10, 36, 62, 72, 180), have also been studied.

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