cating the step in the reaction sequence and superscripts (H, PO_4, $H_2PO_4^-$, HPO_4^{2-}) denoting the catalyst, if any. PO_4 is total phosphate. K_{hyd}(unitless) is [II H_2O]/[II] in water.
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Intramolecular Effects of Radioiodine Decay in *o*-Iodophenol, a Model for Radioiodinated Proteins[1]

Victor W. Jiang,[2] Kenneth A. Krohn,[3] and Michael J. Welch*

Contribution from Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received December 23, 1974*

Abstract: As a model for the chemical effects of the decay of radioiodine in iodinated proteins, [^{131}I-^{14}C]-*o*-iodophenol has been synthesized and the iodine-131 allowed to decay in aqueous solutions containing various phenolic additives. Reference samples were also studied in which the radioiodine was added as $^{131}I^-$ to [^{127}I-^{14}C]-*o*-iodophenol. Similar products were observed from hydrolytic deiodination of the *o*-iodophenol, radiolysis of the molecule by external radiation, and as a result of decay of organically bound iodine-131. A mechanism is proposed in which decay of the iodine in [^{131}I-^{14}C]-*o*-iodophenol leaves a carbonium ion [$C_6H_4(OH)^+$] which in solution reacts to form catechol. It is postulated that the other products formed are oxidation products of catechol.

Although proteins have been radiolabeled with tritium[4,5] and carbon-14,[6] radioiodinated proteins have also been extensively used for both in vivo[7] and in vitro tracer studies.[8] In these studies an iodine atom is substituted for a hydrogen atom so that it is important to know that the molecule retains its biological activity. It has been suggested that one iodine atom on a protein molecule does not alter biological activity, but overiodination correlates with loss of activity.[9] In a protein iodinated at a high specific activity, there is a high probability that one protein molecule will contain more than one atom of radioiodine. Oncley has proposed a binomial formulation for the distribution of iodine atoms attached to a population of molecules.[10] Rosa et al. applied this mathematical treatment to human serum albumin with the prediction that at radioiodine levels of 0.5–1 atom per protein molecule, about 39 and 60%, respectively, of the iodine atoms are bound to molecules containing more than one iodine atom.[11] This theory presents a lower limit to the degree of multiple iodination in that anything less than perfect mixing would lead to regions with even more molecules containing more than one iodine atom. Therefore, at high levels of iodination there is a high probability that one radioiodine atom in a molecule will decay and leave the molecule still labeled. This nuclear decay process results in the release of very large amounts of energy to be distributed between the leaving nuclear particles and the residual heavy nucleus. In order to determine the molecular alterations caused by such a decay and to assess the significance of this local damage on the properties of a molecule, we have initiated a program to assess the chemical effects of iodine decay in model compounds.

The major site of iodination in proteins is at the tyrosine residue[12] to form 3-iodotyrosine which is a substituted *o*-iodophenol. Our initial studies have centered on the intramolecular effects of radioiodine decay in *o*-iodophenol. The method used to study this effect was to prepare doubly labeled [^{131}I-^{14}C]iodophenol and to measure the ^{14}C-labeled residues after β^- decay of the ^{131}I. In a comparison study high specific activity [^{127}I-^{14}C]iodophenol was prepared and the products of the decomposition of this molecule were measured. Any decomposition observed will be a product of hydrolytic deiodination caused by the weak carbon-iodine bond strength and radiolysis due to the effect of extramolecular radiation.

Methods. High specific activity (35 mCi/mmol) uniformly labeled [^{14}C]phenol (0.55 ^{14}C atoms/phenol molecule) (Amersham Searle Corp., Arlington Heights, Ill.) was iodinated with "carrier-free" protein iodination grade iodine-131 (Industrial Nuclear Corp., St. Louis, Mo.) using the chloramine-T method of iodination.[13] Phenol (10 μCi of carbon-14) was allowed to react with 50 mCi of iodine-131 at pH 8.5 using 100 μg of chloramine-T. The iodinated phenols were separated by liquid chromatography [Sephadex G-10-120, bed volume 15 ml, eluted with ammonium hydroxide–ammonium chloride (buffer 0.0025 *M*, pH 9.4) at 40 ml/hr]. In the labeling experiment only the *o*- and *p*-iodophenols were observed, and a good separation of the doubly labeled iodophenol was obtained. Labeling yields based on ^{131}I incorporation of >60% were obtained, of which ~70% was *o*-iodophenol. The *o*-iodophenol was stored at 2–4° at an initial concentration of 0.5 mCi/ml (0.5 mCi of ^{131}I/ml of $\sim 10^{-8}$ *M* iodophenol) containing 0.1 *M* ethanol and dissolved nitrous oxide as scavenger to reduce the amount of radiolysis. The pH was maintained at 7.0 using a

Table I. Uv Absorbance Data and Radioactivity Data Used to Identify Catechol

Fraction no.	Uv absorbance, 280 mμ	^{14}C (cpm) β scintillation	Ratio (uv/^{14}C)
18	0.020	9.5	0.00210
19	0.058	27.1	0.00215
20	0.162	80.4	0.00201
21	0.156	73.6	0.00212
22	0.087	44.0	0.00197
23	0.052	23.4	0.00220

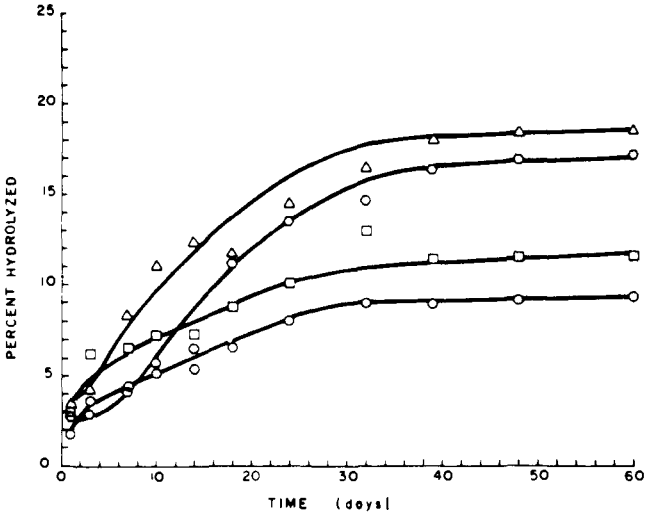


Figure 1. Amount of ^{131}I from [^{131}I-^{14}C]-*o*-iodophenol as a function of time. All preparations contain 0.1 *M* C_2H_5OH and N_2O. Other additives are △, no additive; ◷, 0.1 *M* phenol; □, 0.1 *M* catechol; ○, 0.01 *M* *o*-iodophenol.

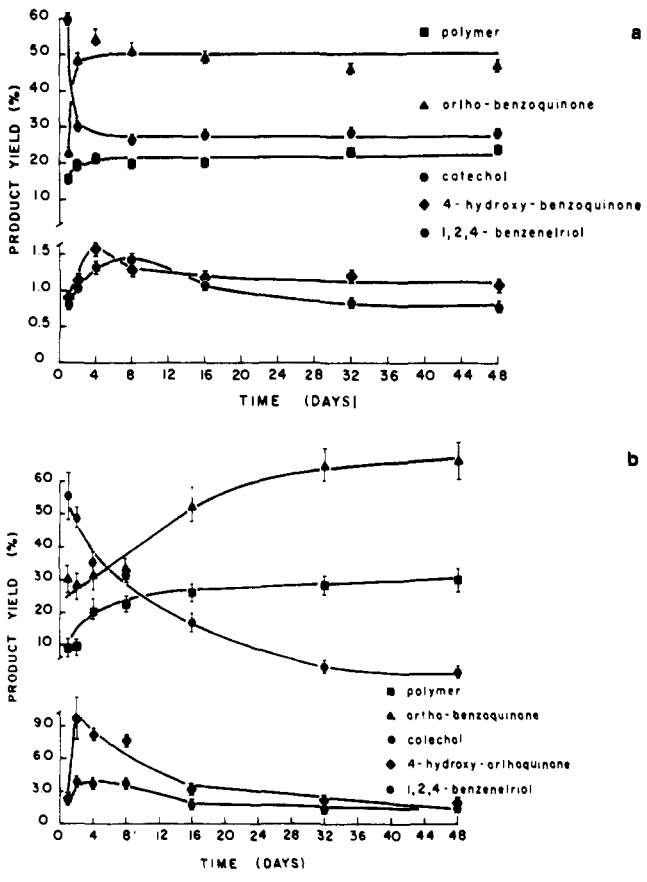


Figure 2. (a) Products observed from the hydrolysis of [^{127}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol as additive: △, *o*-benzoquinone; ◯, catechol; ◇, 4-hydroxy-*o*-quinone; ○, 1,2,4-benzenetriol; □, polymer. These same symbols are used in Figures 2b–5b. (b) Products observed from the β^- decay of ^{131}I in [^{131}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol as an additive.

0.005 *M* ammonium hydroxide–ammonium chloride buffer. Appleby and Umfrid[14] have shown that the mixture of N_2O–ethanol is a good combination for protecting iodinated substrates from decomposition. In other samples carrier phenol, catechol, and *o*-iodophenol as well as the N_2O–ethanol scavenger were added to investigate the effect of additives upon the product spectra. With the exception of *o*-iodophenol, 0.1 *M* concentration of additive was used. Due to the limited solubility of *o*-iodophenol, a 0.01 *M* solution was employed. The release of free iodide from *o*-iodophenol was monitored by withdrawing small aliquots of the solution and measuring the fraction of activity present as ^{131}I using a very short Sephadex G-10-120 column. The hydrolytic deiodination of samples was also studied with [^{131}I-^{12}C]-*o*-iodophenol, where the amount of ^{131}I used was $^1/_{100}$ that used in the doubly labeled experiment, to assess the amount of hydrolytic deiodination at very low levels of radiation.

To determine the products formed by hydrolytic deiodination of iodophenol, duplicate samples were prepared containing a concentration of [^{127}I-^{14}C]-*o*-iodophenol equal to that of [^{131}I-^{14}C]-*o*-iodophenol used in the doubly labeled experiment, the same concentrations of additives, and the same amount of ^{131}I. In this case the ^{131}I was present as iodide ion, and the samples were analyzed in the same manner as the doubly labeled samples purely to differentiate the effects of hydrolytic deiodination and radiolysis.

Sample Analysis and Product Identification. After varying times the solutions were separated and fractions collected by liquid chromatography (Sephadex G-25-40, bed volume 15 ml, eluted with distilled water at 10 ml/hr), and the ^{14}C-labeled decomposition products were determined by liquid scintillation counting after the ^{131}I had decayed for at least 10 half-lives. In this separation the Sephadex is not working in the gel-permeation mode; the compounds are being chemically adsorbed.[15] The products observed were *o*-benzoquinone, catechol, 4-hydroxy-*o*-quinone and 1,2,4-benzenetriol. Varying amounts of a peak eluted at the void volume were observed, which is probably polymeric material. The products were identified by adding known carrier compounds to solutions containing ^{14}C-labeled decomposition products, collecting the individual peaks in at least five aliquots, and determining the specific activities. This method of product identification was chosen as it is very accurate, and under certain conditions differences in specific activities have been obtained using this technique.[16] Catechol (Fisher Scientific) and 1,2,4-benzenetriol (Eastman Organic Chemicals) were obtained commercially, and the 4-hydroxy-*o*-quinone was prepared by the oxidation of 1,2,4-benzenetriol using hydrogen peroxide.[17]

One of the major products is identified as *o*-benzoquinone, a product known to readily undergo polymerization.[18,19] *o*-Benzoquinone was prepared by the oxidation of catechol either with $NaIO_4$ in aqueous solution or Ag_2O in ether.[20,21] Immediately after oxidation the product had the same retention time as the ^{14}C compound. If the authentic compound was allowed to stand or was refluxed in ether, a dimer (or polymer) was formed,[19] which eluted at the void volume on the chromatographic system. When the freshly prepared *o*-benzoquinone was separated by liquid chromatography, and then reduced with lithium aluminum hydride, only catechol was observed. When the dimer was prepared and reduced in the same manner, no catechol was ob-

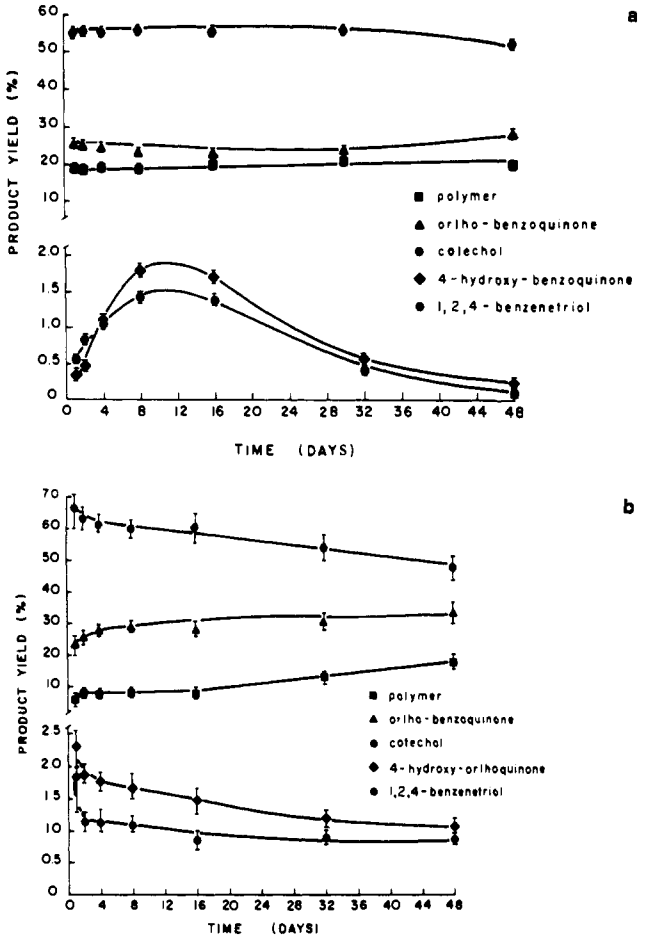


Figure 3. (a) Products observed from the hydrolysis of [^{127}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol and 0.1 *M* phenol as additives. (b) Products observed from the β^- decay of ^{131}I in [^{131}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol and 0.1 *M* phenol as additives.

served. At the very low concentrations used in these experiments dimerization would be slow. The peak at the void volume probably results from Diels–Alder polymerization of products.

Results

The products were identified as discussed in the Methods section, the major products from all experiments being the polymer, *o*-benzoquinone, catechol, 4-hydroxy-*o*-quinone, and 1,2,4-benzenetriol. The data used to identify catechol are given in Table I, where the specific activities of all the aliquots are in good agreement. Similar correspondence between the uv and radioactivity data was found for all the peaks identified.

Figure 1 shows the time-dependent release of ^{131}I ion from the four samples containing identical amounts of doubly labeled *o*-iodophenol but with different additives. As a comparison, in three dilute samples containing 1% the amount of ^{131}I labeled [^{12}C]iodophenol, where very little radiolysis would be expected, the amount of hydrolytic deiodination occurring in 60 days was 9 ± 2%.

Figures 2a–5a show the time-dependent yield of products formed by hydrolytic deiodination and radiolysis in the four systems studied, as determined from the samples containing [^{127}I-^{14}C]-*o*-iodophenol and ^{131}I. The yields of products observed are expressed as

$$\frac{100 \times \%\text{ of product}}{100 - \%\text{ of } o\text{-iodophenol not hydrolyzed}}$$

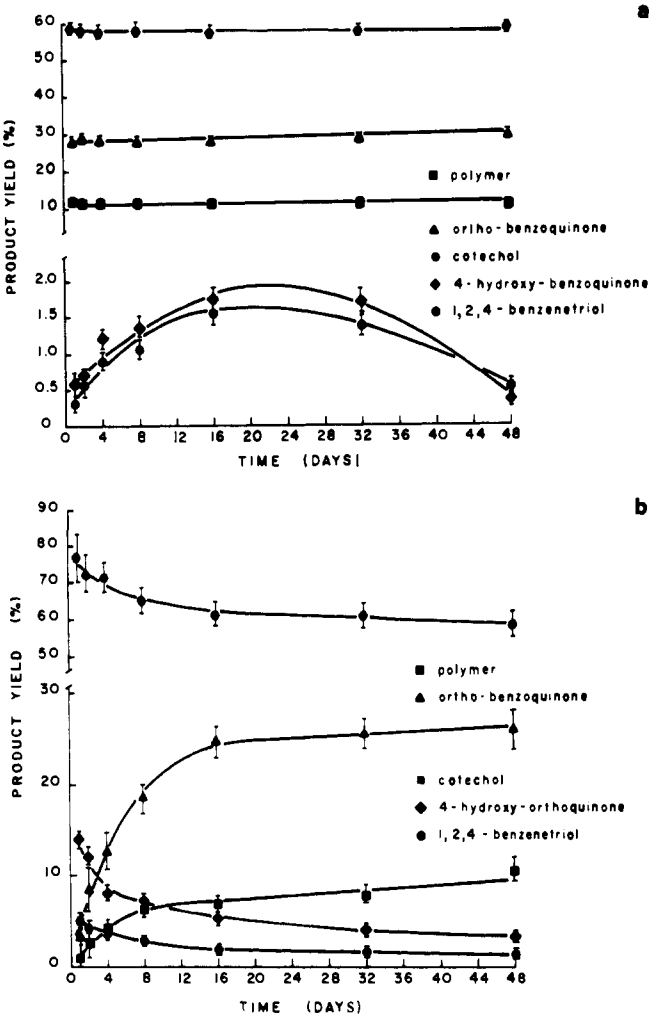


Figure 4. (a) Products observed from the hydrolysis of [^{127}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol and 0.1 *M* catechol as additives. (b) Products observed from the β^- decay of ^{131}I in [^{131}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol and 0.1 *M* catechol as additives.

These yields are then a measure of the change of product produced by hydrolytic deiodination and radiolysis with time. Figures 2b–5b show the data for the doubly labeled experiment where the concentrations were the same as in the others, but the ^{131}I is chemically bound to the phenol. In these data corrections have been made for the hydrolytic deiodination and radiolysis by creating the yields given in Figures 2b–5b according to the formula

$$\%\text{ product} = \frac{100\ (\%\text{ product observed} - \%\text{ product in equivalent } [^{127}\text{I}]\text{-iodophenol sample})}{100 - (\%\ o\text{-iodophenol} + \%\text{ deiodination})}$$

The numerator corrects the percent of a particular product formed by decay by subtracting the amount formed via hydrolytic deiodination, and the denominator is simply the total amount of products formed after ^{131}I decay.

In Figures 2b–5b we are therefore only showing the amount of product that can be attributed to the decay of ^{131}I bound to phenol in the doubly labeled molecules. In all cases ≥90% of the activity was eluted from the column when compared to a standard aliquot of the sample. The amount observed was slightly (~2–3%) greater in the case of the singly labeled samples than the doubly labeled samples.

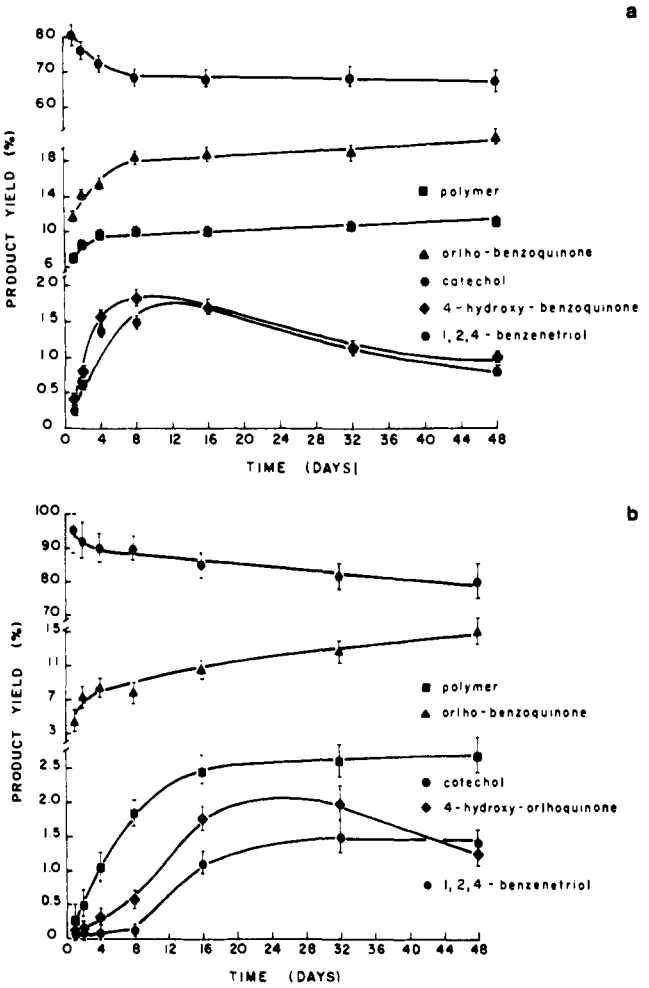


Figure 5. (a) Products observed from the hydrolysis of [^{127}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol and 0.01 *M* *o*-iodophenol as additives. (b) Products observed from the β^- decay of ^{131}I in [^{131}I-^{14}C]-*o*-iodophenol solution containing 0.1 *M* ethanol and 0.01 *M* *o*-iodophenol as additives.

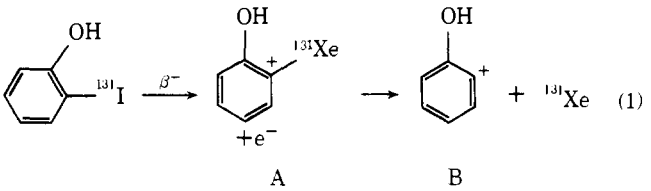
Discussion

The rate of production of I^- from the samples is less than ~1/3% per day in the worst case. Following an initial fast 2-3% hydrolytic deiodination per day, probably caused before dilution and addition of the ethanol-N_2O scavenger, the amount of hydrolytic deiodination with *o*-iodophenol and catechol additives is similar to the case where there is very low radiation dose (9 ± 2% in 60 days). In the other two series the hydrolytic deiodination is slightly greater, the extra hydrolysis presumably being a radiation effect. The amount of I^- released is less than that observed by other workers using similar compounds at low radiation dose.[22]

The observation that the amount of I^- released reaches a plateau value has been noted previously.[22] No satisfactory explanation has been given, especially as at $<10^{-8}$ *M* it is doubtful if the reverse reaction of $RI + H_2O \rightleftarrows ROH + HI$ would occur. In the present study, however, the hydrolytic deiodination data are used primarily to correct data for this effect.

The products observed in the two cases (no additive and phenol additive) where there is apparently some radiolytic deiodination are qualitatively the same as in the two cases where there appears to be no radiolytic deiodination. It appears therefore that the extramolecular radiation simply accentuates hydrolytic deiodination and does not lead to new products.

Hydrolytic deiodination and decay of ^{131}I also qualitatively give the same products: catechol, *o*-benzoquinone, 1,2,4-benzenetriol, 4-hydroxy-*o*-quinone and polymeric material, the latter being of unknown composition, eluting at the void volume of the gel. The ^{131}I nuclide decays by electron emission[23] to form the daughter nuclide ^{131}Xe and the carbon-xenon bond formed is quickly broken due to the intrinsic weakness of that bond. The energy released is distributed according to the laws of energy and momentum conservation between the leaving electron and the recoiling xenon nucleus (eq 1). The eventual formation of the carbo-



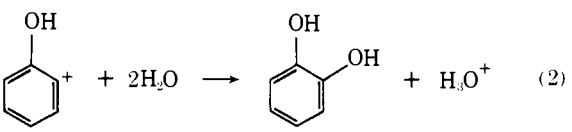
(1)

nium ion $C_6H_4(OH)^+$ is to be anticipated from the mass spectrometric work of Carlson and White,[24] who observed ions in the form $C_xH_y^+$ as the major product from the decay of $C_xH_y{}^{131}I$ species. The positive charge will presumably be left on the carbon atom,[25] and the whole molecule should behave as an aromatic carbonium ion. This is also in agreement with Carlson's mass spectrometric studies on the fragments formed by the β^- decay of tritium in C_6H_5T.[26] In this study the primary $C_6H_5He^+$ decomposed to the $C_6H_5^+$ species in ≃70% of the decay events, the remainder leading to smaller hydrocarbon fragments. The C_6H_5T studies were carried out in the gas phase at low pressure. In the liquid matrix one would expect an even greater fraction of the parent ions (B) to be stabilized; intermediate species (A) will presumably have a short lifetime due to the weak C-Xe bond strength.

It is seen from Figures 2-5 that the hydrolytic deiodination products and the products formed after β^- decay of ^{131}I are qualitatively the same, the differences being that in the absence of decay effects there is less product variation with time. This can perhaps be explained by the fact that hydrolytic deiodination continues with time, whereas a large fraction of the decay process has occurred after short times. In one case we are looking at the continual generation of an intermediate, whereas in the other the intermediate is largely formed initially and then is present in the solution for a longer time. All the compounds identified can be accounted for as oxidation products of catechol, possibly oxidized by hydrated electrons or hydrogen peroxide formed radiolytically[27] or by traces of dissolved oxygen in the solution.

Catechol is easily oxidized by hydrogen peroxide to form *o*-benzoquinone[21] which is less polar than phenol and is eluted quickly from the chromatographic column. Hydrolysis of *o*-benzoquinone produces 1,2,4-benzenetriol[28] which has a long retention on the chromatographic column, probably because of the effect of the three hydroxyl groups.

The product eluted from the chromatographic column at the void volume is a polymer product of various chemical species and different degrees of polymerization. The great yield of catechol observed in the experiment when phenolic compounds are added is plausible because phenol can act as a scavenger for species formed in the radiolysis of water,[29] thereby protecting the labeled catechol, once formed, from further oxidation (eq 2-8). It appears likely, therefore, that



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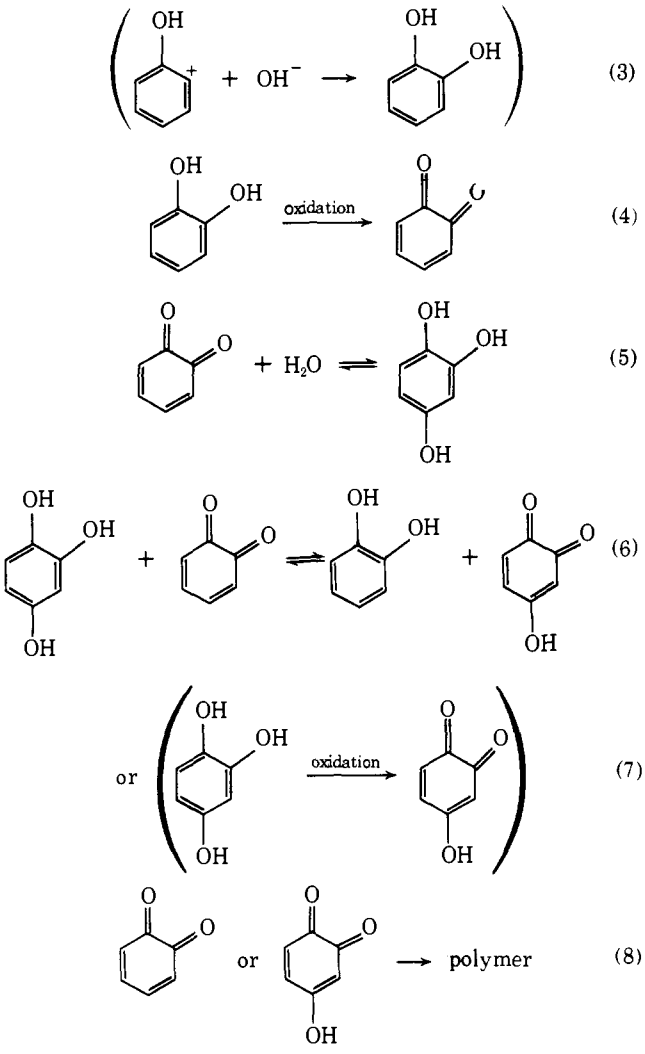
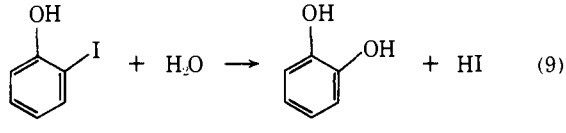


the major reaction following the decay of an iodinated phenol is the formation of a second hydroxyl group on the benzene ring, followed by oxidation in the aqueous media.

The primary reaction observed here is quite similar to that observed by Nefedev et al.[30] studying the carbonium ion CT_3^+ formed by the decay of CT_4. In the presence of water, methanol, or ethanol in the gas phase they measured yields of CT_3OH of between 80 and 90%. They suggest an initial mechanism of the formation of a charged intermediate of the type CT_3-O^+HR which collapses to methanol through an unspecified reaction with the inactive hydroxylic compounds. The direct pathway of the type $CT_3OH_2^+ + H_2O \rightarrow CT_3OH + H_3O^+$ can be ruled out, unless the $CT_3OH_2^+$ is still excited, as this reaction is endothermic by about 12 kcal/mol.[31] To form catechol we would postulate the following reaction in the case of hydrolysis.



The reaction can be estimated to be approximately thermoneutral ($\Delta H \simeq 0.02$ eV).[32,33] This reaction would be expected to be very pH sensitive. This has been shown to be the case[34] with the relative amounts of hydrolytic deiodination in 60 days at pH's 9, 7, and 2 being 1.62:1.29:1. If the carbonium ions are first formed by β^- decay of the attached ^{131}I, the possible reactions 2 and 3 would be exothermic by approximately 13 and 7 eV, respectively. These reactions are consistent with the very slow hydrolytic deiodination of the *o*-iodophenol and the very high yield of catechol-related products observed in all cases. As the total activity observed in both series of experiments was very high, only a small fraction of the reactions could lead to products not identified in these studies. It has been mentioned that a small percent more activity was observed in the hydrolytic deiodination studies. This difference is the maximum of the initial intermediate that could be of a completely different form, possibly breaking the aromatic ring, and lead to products not observed here. To date no authentic compounds have been found that do not elute from the column in the maximum volume used.

Inspection of Figures 2–5 shows that the amount of catechol present at any time is greatest with *o*-iodophenol additive and decreases from the situation with catechol additive to that with phenol additive to the situation with no additive. The amounts of *o*-benzoquinone and polymer increase in all cases, while the amounts of the two minor products peak and then decrease consistent with the mechanism we have discussed.

Hydrolytic deiodination, radiolysis, and β^- decay in labeled *o*-iodophenol all apparently lead to the same primary product, catechol. This product can be protected by addition of various phenols, the efficiency being iodophenol > catechol > phenol. This is the order of decreasing pK_a[35] of the additives. Addition of the stronger acids might be expected to influence the rate of reaction 9 and iodophenol and catechol, being easily decomposed, will perhaps act as very good scavengers for primary radiolysis products. These additives have the same qualitative effect on the hydrolysis of *o*-iodophenol to the catechol.

If our work on this simple model compound can be extended to predict the behavior of labeled proteins, it would suggest that the effect of the decay of an iodine bound to a tyrosyl residue will be to convert the tyrosyl residue to a 3,4-dihydroxyphenylalanyl residue. This may then suffer oxidation even at very low radiation levels.

It is unlikely that inferences to the behavior of iodine-125 labeled proteins can be drawn from this work, as iodine-125 decays by electron capture and internal conversion. Both of these processes involve the formation of K and L shell vacancies in atoms undergoing decay and lead to extensive Auger electron cascades.[36] In this case, the transformation is $^{125}I \rightarrow {}^{125}Te^{n+}$ + electrons and the electronic excitation will be considerably greater, so that it is possible that rupture of the aromatic ring may occur.

Absorption studies of X-radiation in solid-phase iodothyronines[37] have shown that resonance absorption in the iodine has a distinct effect upon the chemical yields. In this case Auger electron cascades predominated, and the authors observed only iodine, iodide, and unidentified amino acids as products. It is possible then that a cage effect will inhibit the fragmentation and in solution that the products from ^{125}I decay will be similar to the ^{131}I case.

It should be noted that the process observed by the decay of iodine-131 bound to an aromatic ring appears to lead to the formation of an aromatic carbonium ion. This is, therefore, a simple method for the preparation of very low concentrations of aromatic carbonium ions in solution similar to that used by Cacace[38] to study simple carbonium ions in the gas phase. In the technique discussed by Cacace, multiply labeled tritium compounds are allowed to decay, where here one isotope of a doubly labeled compound is allowed to decay, allowing a greater range of product identification.

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References and Notes

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Porphyrin–Protein Bond of Cytochrome c. Structure of Porphyrin c

James T. Slama, Herman W. Smith, C. Grant Willson, and Henry Rapoport*

Contribution from the Department of Chemistry, University of California, Berkeley, California 94720.

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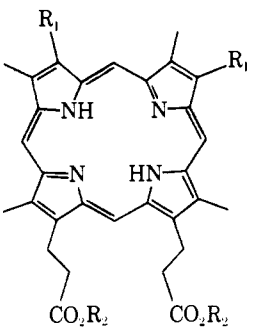
Abstract: Porphyrin c is the porphyrinic substance obtained from acid hydrolysates of cytochrome c. This substance, isolated from both yeast and horse heart cytochrome c, is shown to be 2,4-di(α-*S*-cysteinylethyl)deuteroporphyrin IX. A series of degradative, synthetic, and NMR studies are presented which conclusively establish the α-thioether linkage of the cysteine residues of the apoprotein to the 2- and 4-ethyl substituents of the porphyrin prosthetic group both in cytochrome c and in porphyrin c.

Cytochromes c are perhaps the most thoroughly investigated of all mitochondrial proteins, and the primary structures of cytochromes c from a wide variety of organisms have been elucidated.[1] The cytochromes c differ from most other hemoproteins in their covalent bonding of the iron-porphyrin prosthetic group to the apoprotein,[1a,2,3] whereas the bonding of protein to metalloporphyrin in other hemoproteins belongs to either the ligand or combined ligand-covalent types.[1a]

An appreciation of the detailed covalent bonding between porphyrin and peptide in cytochromes c has accrued over a 40-year period, beginning with the observation that acid hydrolysis of horse heart cytochrome c gave a homogeneous, amphoteric porphyrin fraction, porphyrin c.[4] This porphyrin c, upon treatment with hydrogen bromide in acetic acid, was converted to hematoporphyrin IX (**1**) which subsequently[5] was converted to mesoporphyrin IX (**2**).[5] These observations clearly established that porphyrin c belongs to the porphyrin III series.

Subsequent investigations,[6,7] including acid hydrolysis, demonstrated that porphyrin c was a dicysteinyl adduct of protoporphyrin IX and structure **3** was advanced. That is, the sulfhydryl groups of the cysteine residues were deduced to be attached to the 2,4-ethyl substituents of the porphyrin via thioether bonds, thus covalently joining amino acid to



1, R_1 = CHOHCH$_3$; R_2 = H
2, R_1 = CH_2CH_3; R_2 = H
3, R_1 = $CH(CH_3)SCH_2CH(NH_2)CO_2H$; R_2 = H
4, R_1 = $CHDCH_3$; R_2 = CH_3
5, R_1 = $CHDCH_2D$; R_2 = CH_3
7, R_1 = $CHBrCH_3$; R_2 = CH_3
8, R_1 = $CH_2CH_2SCH_2CH(NHCOCH_3)CO_2CH_3$; R_2 = CH_3
9, R_1 = $CH(CH_3)SCH_2CO_2CH_3$; R_2 = CH_3

porphyrin. However, the reservation was expressed[6b] that porphyrin c obtained by these hydrolytic methods may not reflect the original cytochrome thioether structure.[6b]

This question of cytochrome c thioether rearrangement