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The Oxidative Cleavage of Tyrosyl–Peptide Bonds during Iodination*

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ABSTRACT: In addition to the usual diiodination of the phenolic ring, *N*-iodosuccinimide is capable of effecting an oxidative transformation of tyrosine models and derivatives by which dienone lactones are formed. Thus, diiodophloretic acid, *N*-carbobenzyloxy-3,5-diiodotyrosine, and *N*-benzoyl-3,5-diiodotyrosine are converted into their respective dienone lactones (**2a–2c**) in yields of 57–95% in acetate buffer, pH 4.5. Similarly, *N*-iodosuccinimide effects the oxidative cleavage of the peptide bond in diiodophloretylglycine and in *N*-carbobenzyloxytyrosylglycine. Because the oxidation potential of iodine is considerably lower than that of *N*-iodosuccinimide, only ring halogenation is observed with the former reagent.

The reactivity of *N*-iodosuccinimide is decreased markedly in the presence of acetonitrile. The possibility of peptide-bond cleavage in the course of protein iodination is considered.

Previous studies in this laboratory (Wilson and Cohen, 1963) have demonstrated the facile cleavage of tyrosyl–peptide bonds in simple peptides and in proteins as a result of the oxidative action of bromine or of *N*-bromosuccinimide. Although positive iodine reagents are generally weaker oxidants than their bromine counterparts, it is entirely reasonable to expect that iodine oxidants of sufficient potential would effect analogous cleavage reactions. The possibility is particularly significant in view of the extensive use of iodination as a technique in protein modification.[1] Such cleavage has, indeed, been found to occur and, in this report, the oxidative fission of simple tyrosyl dipeptides by *N*-iodosuccinimide is described.

Experimental Section

Iodination of Phloretic Acid with N-Iodosuccinimide. To a stirred solution of 166 mg (1 mmole) of phloretic acid (3-*p*-hydroxyphenylpropionic acid, Aldrich Chemical Co.) in 10 ml of acetonitrile and 40 ml of acetate buffer (0.2 M, pH 4.5) was added 900 mg (4 mmoles) of *N*-iodosuccinimide in one portion. After 20-min stirring of the reaction mixture in the dark, the crystalline precipitate was collected, washed with 40% acetone–water and with carbon tetrachloride, and dried, 295 mg, 71%. The dienone lactone (**2a**) was recrystallized from acetonitrile–water: mp 223–226°,[2] ultraviolet maximum (ethanol) 295 mμ (ε 3120) and 265 mμ (ε 3560), and infrared spectrum (KBr) 1780 (lactone C=O) and 1670 cm^{-1} (dienone C=O).

Anal. Calcd for $C_9H_6I_2O_3$: C, 25.99; H, 1.45; I, 61.02. Found: C, 26.24; H, 1.94; I, 61.18.

Iodination of phloretic acid with excess iodine gave 3,5-diiodophloretic acid (**1a**) (Barnes *et al.*, 1950): (a) iodine–potassium iodide, Tris buffer, pH 8.9, 45% yield; (b) iodine–chloroform, phosphate buffer, pH 6.8, 75%; (c) iodine–acetonitrile, acetate buffer, pH 5.4, 41%. In no case could **2a** be detected in these iodinations, either by thin-layer chromatography or following work-up.

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[1] For a recent review, see Cohen (1968).

[2] Matsuura *et al.* (1967) report mp 225–26°.

Oxidation of 3,5-Diiodophloretic Acid (**1a**) *with N-Iodosuccinimide.* To a suspension of 209 mg (0.5 mmole) of diiodophloretic acid in 20 ml of acetate buffer (0.2 M, pH 4.5) was added 250 mg (1.1 mmoles) of *N*-iodosuccinimide. The mixture was stirred 20 min (dark), and the precipitate was collected and recrystallized from acetonitrile–water to give 196 mg (95%) of **2a**. When the oxidation was performed in an acetate buffer–acetonitrile mixture (1:1, v/v), the yield was reduced to 63%.

Diiodophloretic acid was recovered unchanged following exposure to iodine solution at several pH values.

Reaction of N-Carbobenzyloxytyrosine with N-Iodosuccinimide. To a stirred solution of 300 mg (0.95 mmole) of *N*-carbobenzyloxytyrosine in 30 ml of acetate buffer (0.2 M, pH 4.5) was added 900 mg (4 mmoles) of *N*-iodosuccinimide in one portion. Precipitation of the dienone lactone (**2b**) began immediately. After 15 min, the precipitate was collected by filtration, washed with water, and crystallized from acetonitrile–water: 303 mg, 57%, mp 195–199°. For analysis, a sample was recrystallized from acetonitrile: mp 195–201°, ultraviolet maximum (ethanol) 295 mμ (ε 3600) and 262 mμ (ε 4180), and infrared spectrum (KBr) 1790 (lactone C=O), 1710 (urethan C=O), and 1672 cm^{-1} (dienone C=O).

Anal. Calcd for $C_{17}H_{13}I_2NO_5$: C, 36.13; H, 2.32; I, 44.91; N, 2.48. Found: C, 36.38; H, 2.46; I, 44.77; N, 2.55.

Reaction of N-Carbobenzyloxytyrosine with Iodine. To a solution of 315 mg (1 mmole) of *N*-carbobenzyloxytyrosine in 15 ml of phosphate buffer (0.2 M, pH 6.8) was added 30 ml of a solution of iodine (4 mmoles) in chloroform. After the mixture had been stirred 2 hr at room temperature, the chloroform phase was separated and the aqueous phase was extracted with five portions of chloroform. The combined chloroform layers were dried (Na_2SO_4) and evaporated to give 200 mg of *N*-carbobenzyloxy-3,5-diiodotyrosine (**1b**) which was recrystallized from benzene–ether: mp 169–173°, ultraviolet maximum (ethanol) 294 mμ (ε 2840) and 287 mμ (ε 3140), and mass spectrum *m/e* 567.

Anal. Calcd for $C_{17}H_{15}I_2NO_5$: C, 36.00; H, 2.67; I, 44.75; N, 2.50. Found: C, 36.20; H, 2.85; I, 44.66; N, 2.50.

Reaction of N-Benzoyl-3,5-diiodotyrosine (**1c**) *with N-Iodosuccinimide.* To a solution of 537 mg (1 mmole) of *N*-benzoyl-3,5-diiodotyrosine in 35 ml of acetate buffer (0.2 M, pH 4.5) and 25 ml of acetonitrile was added 450 mg (2 mmoles) of *N*-iodosuccinimide. The mixture was stirred 5 min and the precipitate was collected and recrystallized from acetonitrile to give 350 mg (65%) of **2c** as colorless needles, mp 216°.

Anal. Calcd for $C_{16}H_{11}I_2NO_4$: C, 35.91; H, 2.07. Found: C, 36.14; H, 2.22.

The dienone lactone **2c** could not be detected following exposure of **1c** to iodine under a variety of pH conditions.

3,5-Diiodophloretylglycine (**3a**). To a cooled suspension of 600 mg of glycine ethyl ester hydrochloride in 30 ml of dichloromethane was added 0.58 ml of triethylamine. The mixture was shaken briefly to effect solution and added to a suspension of 1.67 g of 3,5-diiodophloretic acid in 40 ml of dichloromethane. After 5-min stirring at room temperature, 840 mg of *N,N'*-dicyclohexylcarbodiimide in 10 ml of dichloromethane was added and the mixture was stirred at room temperature for 18 hr (dark). The reaction mixture was chilled for 3 hr, the precipitate was removed by filtration, and the filtrate was washed successively with 1 N HCl, 5% sodium bicarbonate, and water. The dichloromethane solution was dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml of methanol and 20 ml of 1.25 N sodium hydroxide was added. After 3 hr at room temperature, a small amount of precipitate was removed from the solution, and the methanol was evaporated *in vacuo*. The aqueous solution was acidified to pH 7 with 6 N HCl and extracted with ether. The aqueous layer was acidified to pH 1 and the crystalline product was collected by filtration, 1.3 g, 68%. The peptide was recrystallized from acetonitrile–water as plates: mp 165–170°, resolidifying as needles and remelting at 182–184°, ultraviolet maximum (ethanol) 294 mμ (sh, ε 2950) and 287 mμ (ε 3240), infrared spectrum (KBr) 1735 cm^{-1} (C=O), and mass spectrum *m/e* 475.

Anal. Calcd for $C_{11}H_{11}I_2NO_4$: C, 27.81; H, 2.33; I, 53.43; N, 2.94. Found: C, 28.01; H, 2.51; I, 52.84; N, 2.88.

Oxidative Cleavage of Diiodophloretylglycine (**3a**) *with N-Iodosuccinimide.* To a solution of 181 mg (0.38 mmole) of diiodophloretylglycine in 20 ml of acetate buffer (0.2 M, pH 4.5) was added 225 mg (1 mmole) of *N*-iodosuccinimide. After stirring the mixture 3 hr at room temperature (dark), it was adjusted to pH 7 with sodium carbonate solution and extracted with several portions of ether. The combined ether extracts were dried ($MgSO_4$) and evaporated to dryness. The residue was dissolved in chloroform and the solvent, together with traces of iodine, removed *in vacuo*. The residue was recrystallized from acetonitrile–water to give 40 mg (25%) of the dienone lactone (**2a**). The presence of glycine in the reaction mixture was demonstrated by paper chromatography. Acidification to pH 1 of the aqueous layer, remaining after ether extraction, resulted in the separation of a solid (75 mg) which resisted efforts at purification or crystallization. On thin-layer chromatography plates, the substance remained at the origin in several solvent systems.

The same cleavage products (**2a** and glycine) were obtained by reaction of phloretylglycine with *N*-iodosuccinimide under similar conditions and by reaction of **3a** with *N*-bromosuccinimide.

Reaction of N-Carbobenzyloxytyrosylglycine with N-Iodosuccinimide. To a solution of 70 mg (0.19 mmole) of *N*-carbobenzyloxytyrosylglycine in 6 ml of acetate buffer (0.2 M, pH 4.5) was added 200 mg (0.89 mmole) of *N*-iodosuccinimide. Precipitate formation began immediately. After storage for 1 hr, the mixture was extracted with ether and the extract was dried ($MgSO_4$) and evaporated. The residue was dissolved in chloroform and the solvent, together with a small amount of iodine, removed *in vacuo*. After recrystallization of the product from acetonitrile, 30 mg (28%) of **2b** was obtained.

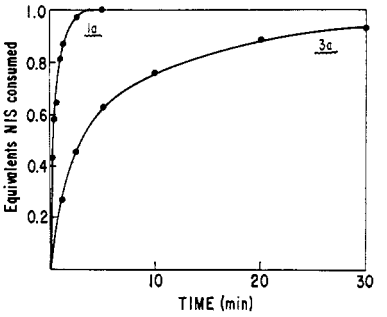


FIGURE 1: Rate of consumption of *N*-iodosuccinimide by diiodophloretic acid (**1a**) and by diiodophloretylglycine (**3a**) in acetate buffer (pH 4.5).

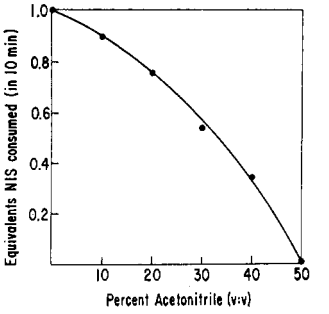


FIGURE 2: Effect of addition of acetonitrile upon the rate of consumption of *N*-iodosuccinimide by diiodophloretic acid (**1a**) in acetate buffer (pH 4.5).

Reaction of N-Carbobenzyloxytyrosylglycine with Iodine. To a solution of 168 mg (0.45 mmole) of *N*-carbobenzyloxytyrosylglycine in 10 ml of phosphate buffer (0.2 M, pH 6.8) was added a solution of 406 mg (1.6 mmoles) of iodine in 12 ml of chloroform. The mixture was stirred 2 hr at room temperature and the precipitate, which had formed in the aqueous phase, was separated, washed with water, and recrystallized from acetonitrile–water to give 60 mg of *N*-carbobenzyloxy-3,5-diiodotyrosylglycine (**3b**): mp 204–206°, and ultraviolet maximum (ethanol) 295 mμ (sh, ε 2850) and 287 mμ (ε 3180). An additional 30 mg of the product was obtained from the chloroform layer. The absence of the dienone lactone **2b** from both phases was demonstrated by thin-layer chromatography.

Anal. Calcd for $C_{19}H_{18}I_2N_2O_6$: C, 36.56; H, 2.91; I, 40.66; N, 4.49. Found: C, 36.77; H, 3.13; I, 40.60; N, 4.54.

Oxidation of N-Benzoyl-3,5-di-t-butyltyrosine (**4**) *with Iodine.* To a solution of 40 mg (0.1 mmole) of *N*-benzoyl-3,5-di-*t*-butyltyrosine (Cohen and Jones, 1962) in 5 ml of phosphate buffer (0.2 M, pH 6.8) was added a solution of 50 mg (0.2 mmole) of iodine in 5 ml of chloroform. The mixture was stirred for 10 min and the chloroform layer was separated, dried (Na_2SO_4), and evaporated to dryness. Excess iodine was removed by washing the residue with hexane until colorless. The crystalline dienone lactone (**5**) was recrystallized from acetonitrile: mp 210–214° dec, ultraviolet maximum (ethanol) 235 mμ (ε 18,200), and mass spectrum *m/e* 395.

Rates of Oxidation. Stock solutions consisted of 104 mg of diiodophloretic acid (**1a**) in 25 ml of ethanol (0.01 M), 116 mg of diiodophloretylglycine (**3a**) in 25 ml of ethanol (0.01 M), and 1.125 g of *N*-iodosuccinimide in 10 ml of ethanol (1 M). To 9 ml of acetate buffer (0.2 M, pH 4.5) was added 1 ml of substrate solution and then 0.05 ml of *N*-iodosuccinimide solution, providing an iodine: substrate ratio of 5:1. After an appropriate time interval, the reaction was quenched by addition of excess potassium iodide–sulfuric acid solution and the liberated iodine titrated with standard thiosulfate. The rates of consumption of *N*-iodosuccinimide by **1a** and **3a** are shown in Figure 1. The results of Figure 2 were obtained in a similar way, varying the ratio of acetate buffer to acetonitrile in the medium.

Chromatographic Data. Thin-layer chromatography was performed on silica gel GF plates, using benzene–dioxane–acetic acid (90:25:4, v/v) as developing agent. R_F values were **1a**, 0.55; **1b**, 0.45; **2a**, 0.62; **2b**, 0.59; **3a**, 0.15; and **3b**, 0.19.

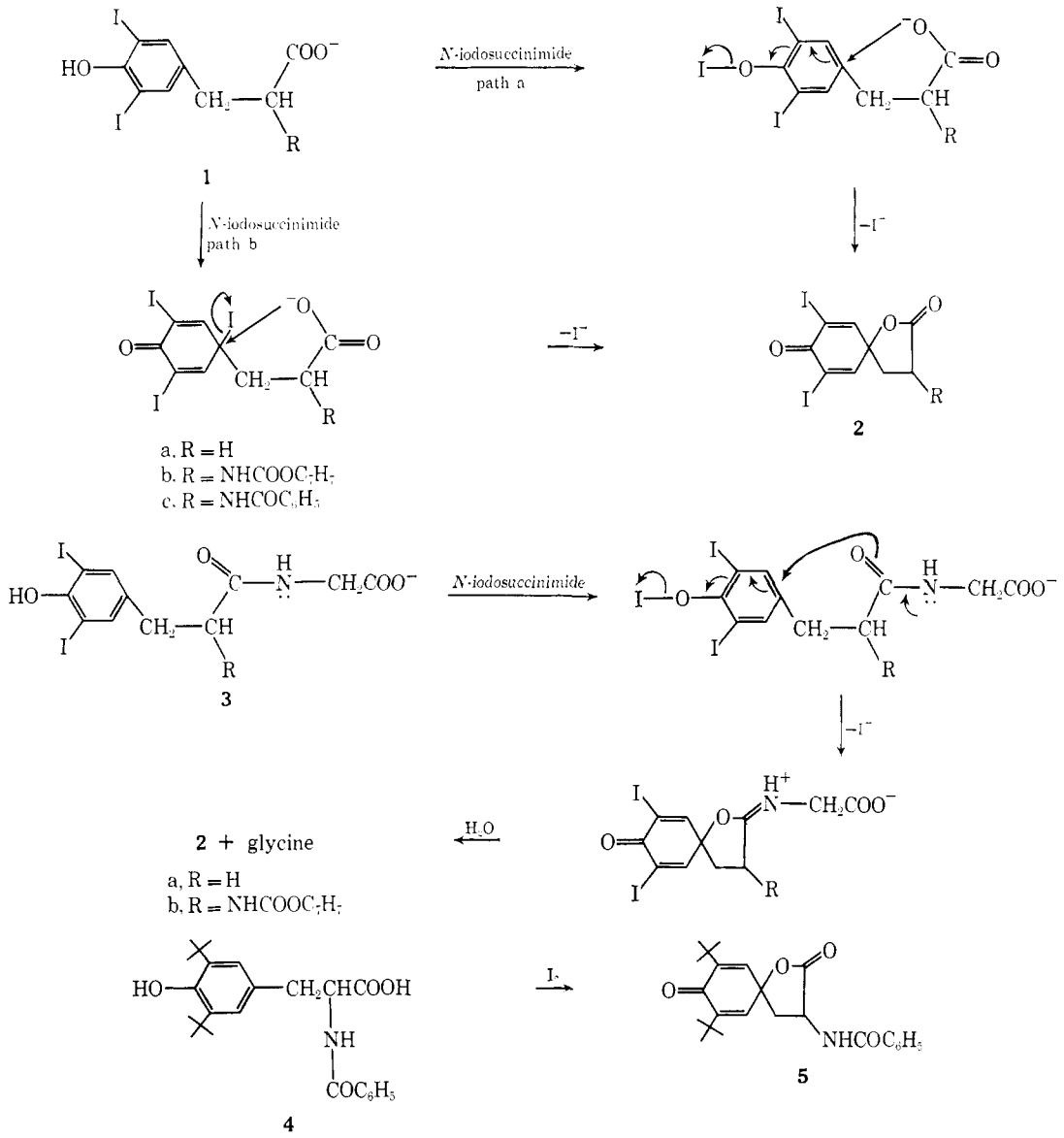
Results and Discussion

By analogy with the oxidation of 3,5-dibromophloretic acid to the corresponding dienone lactone with *N*-bromosuccinimide or bromine, 3,5-diiodophloretic acid (**1a**) (Scheme I) is oxidized to **2a** by *N*-iodosuccinimide or *N*-bromosuccinimide. Although iodine is equally capable of halogenating the phenolic ring, its potential is evidently too low to effect subsequent oxidation of the diiodophenolic ring system. The introduction of the two iodine atoms is sufficient to place the phenolic system beyond the capabilities of iodine as oxidant. On the other hand, the di-*t*-butylphenolic system of **4**, having electron-releasing rather than electron-withdrawing substituents, is readily oxidized by iodine to the dienone lactone, **5**. Whether *N*-iodosuccinimide acts directly, either in halogenation or oxidation, or *via* its hydrolysis product, hypoiodous acid (Seliwanoff, 1893), is not clear at present.

In those cases in which the dienone lactone is formed by participation of a carboxylate ion (**1a–3a**), yields are generally high (65–95%). On the other hand, requisite participation of the amide group (**3a,b**) results in a lower yield of dienone lactone (25–30%). It is evident from Figure 1 that the rate of reduction of positive halogen by diiodophloretic acid (**1a**) is significantly greater than in the case of diiodophloretylglycine (**3a**). Since any iodinated intermediate (path a or b) would still titrate as positive halogen (*cf.* Cook *et al.*, 1955), the kinetics reflect the rate of breakdown of such an intermediate. Furthermore, it is reasonable to assume that the rates of formation of an iodinated intermediate by **1a** and by **3a** would be very similar. If oxidation and participation were coupled in a concerted process, the removal of electrons from phloretic acid should be assisted by carboxyl participation; however, the critical oxidation potential for phloretic acid is very similar to that for *p*-cresol.[3] A more likely explanation is that, as the nucleophilicity of the participating group is re-

[3] J. W. Thanassi and L. A. Cohen, unpublished data.

SCHEME I



duced from that of carboxylate to that of amide, the rate of breakdown of an iodinated intermediate is decreased; resulting competitive attack on the intermediate by external nucleophiles, such as water or buffer ion, would also explain the reduced yields of lactone. A similar competition has been demonstrated to occur during the electrolytic oxidation of tyrosine peptides (Farber and Cohen, 1966; Isoe and Cohen, 1968). Efforts to isolate acidic by-products, *e.g.*, an acetoxy- or hydroxydienone, produced only intractable material which could not be characterized.

In early experiments, it was observed that the rate of consumption of positive halogen (from *N*-iodosuccinimide) by diiodophloretic acid and other substrates was significantly lowered by addition of acetonitrile (spectral or anhydrous grade) to the reaction mixture (Figure 2). No inhibitory effect was observed when *t*-butyl alcohol or tetrahydrofuran was employed as cosolvents. Furthermore, at fixed acetonitrile concentrations, the inhibitory effect increased as pH was raised from 2 to 8. We tentatively consider the effect to be due to the rapid conversion of the strong oxidant, *N*-iodosuccinimide or hypoiodous acid, to the relatively weak oxidant, iodate ion, promoted by traces of ammonia in the solvent or, less likely, by the acetonitrile itself. Indeed, inhibitory effects of comparable magnitude were observed upon addition of traces of ammonium acetate to the normal sodium acetate buffer medium.

It would appear, from these results, that the danger of effecting cleavage of tyrosyl–peptide bonds, in the course of iodination of polypeptides or proteins with iodine, is not serious. On the other hand, when more potent reagents, such as *N*-iodosuccinimide, are employed for iodination, a routine check for the appearance of new

amino terminals may be advisable. In addition to its action on tyrosine, iodine converts imidazole in proteins to mono and diiodo derivatives (Cohen, 1968) and, in certain cases, oxidizes the indole nucleus to an oxindole (Hartdegen and Rupley, 1967). *N*-Bromosuccinimide has been shown to cleave tyrosyl-peptide bonds (Wilson and Cohen, 1963) as well as those of tryptophan and histidine (Witkop, 1961). Whether *N*-iodosuccinimide has a similar action on proteins is currently under investigation.

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Circular Dichroism of Isolated and Recombined Hemoglobin Chains*

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ABSTRACT: The optical activity of the heme bands of hemoglobin has been used as a probe to study the alterations of the structure of the isolated α and β chains on recombination to form the normal tetrameric molecule. In all oxidation and ligand states, the circular dichroism spectra of the two chains differ both in the Soret and in the visible region and are readily distinguishable from each other by this technique. The circular dichroism bands exhibit distinctive peak positions, molar ellipticities, and rotational strengths, reflecting a difference in the environments of the heme in the α from that of the β chains. Chain recombination induces a circular dichroism spectrum identical with that of native hemoglobin, but different from that of the noninteracting mixture, indicating an alteration of the heme environments upon recombination. The largest variations are observed upon recombination of the deoxy chains. Chain recombination when studied in the region of absorption of the peptide chromophore does not appear to indicate a change in the conformation of the molecule except in the case of the deoxy derivative, where there is an increase in the depth of the 233-nm Cotton effect trough comparable with that observed upon deoxygenation of hemoglobin.

The ligand binding properties of the isolated subunits of hemoglobin change dramatically upon recombination, and the properties of the recombined material are identical with those of the native molecule (Antonini *et al.*, 1965, 1967). The physicochemical basis of these phenomena has been investigated by a variety of techniques, and recent studies have demonstrated that some of the spectral properties of the isolated chains differ from those of the native and of the reconstituted molecules. The molar absorptivities in the Soret region of both the α and the β chains in the deoxy form are lower than that of deoxyhemoglobin (Benesch *et al.*, 1964; Antonini *et al.*, 1965), and absorptivity increases when the chains are mixed (Antonini *et al.*, 1966; Brunori *et al.*, 1968). Brunori *et al.* (1967) demonstrated that the amplitude of the Cotton effect trough at 233 nm increases upon deoxygenation of hemoglobin and concluded that the change is dependent upon chain interaction since no change occurs with the isolated *p*-hydroxymercuribenzoate subunits. Beychok *et al.* (1967) found that the α and β chains have different molar ellipticities both in the 260-nm region and between 410 and 415 nm while hemoglobin has intermediate values. Moreover,

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