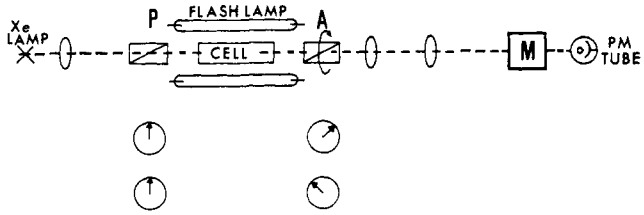

Figure 1. Block diagram of flash spectropolarimeter.

monochromatic by M, a Zeiss MM12 double monochromator. Transient output from the 9558B or Q photomultiplier tube, corresponding to a change in transmitted light, is dc coupled through a cathode follower amplifier to an oscilloscope and photographically recorded. The cylindrical quartz reaction cell (10-cm light path, 2.5 cm i.d.) has evacuated 2-cm cylindrical chambers at each end; surrounding the cell compartment and approximately 75% of each end section is an annular jacket through which is circulated the thermostated saturated aqueous nickel(II) chloride filter solution.

The intensity of light transmitted by the polarizer and analyzer at an angle of rotation θ relative to each other is

$$I = I_0 K \cos^2(\theta + \alpha) \qquad (1)$$

where I_0 is the incident light, K is a constant for the apparatus and absorption cell which accounts for all light losses by reflection, etc., and α is the rotation of the solution. In practice, the observed change in transmittancy following initiation of the flash results from scattered flash light and absorbance changes as well as from rotational change. Accordingly, transmittancy change measurements as a function of time are made with the analyzer rotated $+\theta'$ and $-\theta'$; the transitory change in rotation at a specific wavelength λ and time t after initiation of the flash, $\alpha_t(\lambda)$, is then given by eq 2 if $\alpha_t(\lambda)$ is small compared to θ'.

$$\alpha_t(\lambda) = -\frac{\Delta I_+ - \Delta I_-}{4I' \tan \theta'} \qquad (2)$$

In this expression $\theta' = \theta + \alpha_0$, where α_0 is the steady (assumed constant) rotation of the solution resulting from ground-state optical activity, ΔI_+ and ΔI_- are the changes in transmittancy at $+\theta'$ and $-\theta'$, respectively, and I' is the steady transmitted intensity at $+\theta'$ or $-\theta'$, measured just prior to flash initiation. The best value for θ' is a compromise between photomultiplier tube shot noise and constant measurement errors; for the conditions of oscilloscope sensitivity, etc., used in this work, the best value for θ' was $\pm 80°$. The transitory ORD curve is thus constructed from the above measurements taken as a function of wavelength.

Short- and long-lived transient species, as well as extensive photochemical decomposition, result from the flash excitation of benzoin (benzene solvent). However, transient optical activity was observed in this work only during the lifetime of the short-lived transient (*ca.* 50-μsec half-life and tentatively identified as a benzoin triplet state), and the solutions became completely inactive (from photochemical decomposition processes) after repeated flashings. (For this reason, data were used from oscillograms taken on freshly outgassed solutions flashed less than four times.)

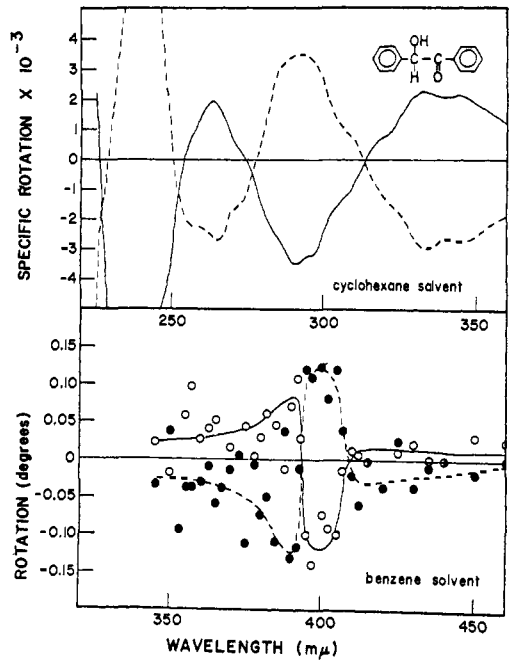

Figure 2. Optical rotatory dispersion curves of the ground and excited states of benzoin: ———, ground-state *d*-benzoin; -----, ground-state *l*-benzoin; —O—, excited state *d*-benzoin; ---●---, excited state *l*-benzoin.

The ORD curves for the excited state, measured at 50 μsec after flash initiation, are given in Figure 2 for both *d*- and *l*-benzoin in the region of triplet–triplet absorption,[3] and are compared with the ground-state ORD curves below 355 mμ obtained on a conventional spectropolarimeter. The experimental accuracy of this technique involving transitory measurements is still relatively low as indicated by the scattering of experimental points. Nevertheless, the excited-state curves are roughly mirror images of each other, and very definite similarities in multiple Cotton effects are shown between the ground and excited states. Electronic excitation not involving bond rupture will not effect the asymmetric environment of the carbonyl chromophore in a molecule such as benzoin; these similarities thus suggest that basic asymmetry and not the specific type of electronic transition may determine the character of the ORD effect.

Work is continuing in this laboratory on related systems.

Acknowledgment. This research was supported in part by the Department of Health, Education, and Welfare, NIGMS Grant No. GM12590.

(3) G. Porter and M. W. Windsor, *Proc. Roy. Soc.* (London), **A245**, 238 (1958).

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The Oxidation by Iodine of Tryptophan 108 in Lysozyme

Sir:

Canfield[1] and Jolles, *et al.*,[2] have shown that the six tryptophans of lysozyme are located at sequence posi-

(1) R. E. Canfield, *J. Biol. Chem.*, **238**, 2698 (1963).
(2) J. Jolles, J. Jauregui-Adell, I. Bernier, and P. Jolles, *Biochim. Biophys. Acta*, **78**, 668 (1963).

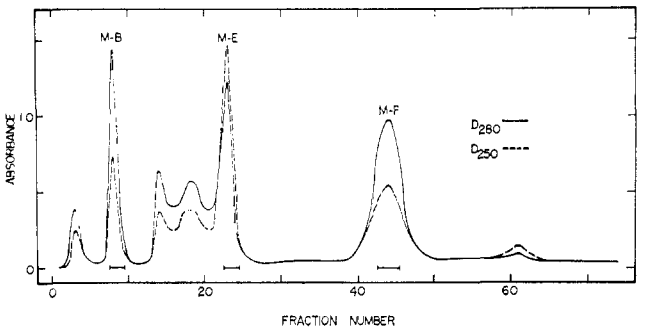

Figure 1. Chromatographic separation of the tryptic peptides of reduced and carboxymethylated iodine-oxidized lysozyme: 17 mg of peptide was applied to a 1 × 30 cm column of Dowex 1, with elution at 0.6 ml/min, using a linear gradient over 200 ml from 0.05 *M* ammonium acetate, pH 8.0, to 2 *M* acetic acid; 3-ml fractions were collected and analyzed for their optical density at 250 mμ (broken line) and 280 mμ (solid line). Oxindole may be distinguished from tryptophan by its higher absorption at 250 than at 280 mμ. Bars indicate fractions pooled for analysis (Table I).

tions 28, 62, 63, 108, 111, and 123. Hayashi and coworkers[3] have demonstrated that N-bromosuccinimide oxidizes tryptophan 62 to the oxindole. The following data show that iodine specifically oxidizes residue 108 in an unexpectedly rapid reaction.

Monooxindole-lysozyme, prepared through reaction with iodine,[4] was reduced,[2] carboxymethylated,[2] and subjected to tryptic hydrolysis (for 2 hr, using 4% chymotrypsin-free trypsin[2] at pH 8 and 40°); the resulting peptides were separated on Dowex-1 (Figure 1). The ultraviolet-absorbing components, MB and ME, were not found among peptides derived from the native enzyme, and their absorption spectra showed that they contained, respectively, tryptophan and oxindoletryptophan. Peptide MF, also present in tryptic hydrolysates of native lysozyme, exhibited a tryptophan spectrum. The fractions corresponding to the components MB, ME, and MF were purified on Sephadex G-25 and analyzed for their amino acid content (Table I). Peptide MF had a composition identifying it as the tryptic peptide, designated T-9 by Canfield[1] or T-17 by Jolles, *et al.*,[2] which contains the sequence tryptophan 62–tryptophan 63, demonstrating that the residue oxidized by iodine is not the one oxidized by N-bromosuccinimide. The composition of peptide ME corresponds to the sequence 98–108, and since the oxindole is in this component, the tryptophan reacting with iodine must be 108. Peptides ME and MB together comprise the normal tryptic peptide designated T-13[1] or T-16,[2] and arise from a split at the carboxyl end of residue 108, which was not observed in control experiments with the native protein. The behavior of the oxindole derivative in sedimentation and dialysis suggests the cleavage occurs at some step subsequent to reduction and carboxymethylation. Apparently, conversion of tryptophan 108 to the oxindole results in a unusual and unexplained sensitivity of the 108–109 bond to either enzymic or nonenzymic hydrolysis. Because of the stability to hydrolysis observed for the 62–63 bond after oxidation of tryptophan 62, the cleavage resulting from oxidation of residue 108 is unusual

(3) K. Hayashi, T. Imoto, G. Funatsu, and M. Funatsu, *J. Biochem.* (Tokyo), 58, 227 (1965).

(4) F. J. Hartdegen and J. A. Rupley, *Biochim. Biophys. Acta*, 92, 625 (1964).

Table I. Amino Acid Composition of Certain Ultraviolet-Absorbing Peptides[a] in the Tryptic Digest of Reduced Carboxymethylated Iodine-Oxidized Lysozyme

Amino acid	MB[b]	Theory[c] for 109–112	ME[b]	Theory[c] for 98–108	MF[b]	Theory[c] for 62–68
Trp or oxindole-alanine	1.00	1	1.00	1	2.00	2
Tyr						
Asp	0.01		3.08	3	2.02	2
Thr						
Ser	0.02		0.95	1	0.03	
Glu	0.01		0.03		0.03	
Gly	0.04		2.13	2	0.97	1
Ala	1.02	1	0.97	1	0.01	
Val	1.00	1	0.31[d]	1	0.04	
Half-Cys			0.08		0.81	1
Met			0.28[d]	1		
Ile			0.27[d]	1	0.01	
Leu	0.01					
Phe						
His						
Arg	1.06	1	0.02		1.03	1
Lys	0.01		0.01			

[a] Isolated from the separation shown in Figure 1. [b] Sample hydrolyzed 18 hr under nitrogen in 6 *M* HCl at 105°. Prior to hydrolysis, norleucine was added in an amount based upon the spectrophotometrically determined content of tryptophan or oxindole-alanine, which are destroyed during acid hydrolysis. From the concentrations of the amino acids relative to norleucine, the amino acid composition of the peptide was calculated from an assumed tryptophan or oxindole content of either 1.00 or 2.00 moles/mole of peptide. [c] From Canfield[1] and Jolles, *et al.*[2] [d] Low recovery probably reflects incomplete hydrolysis of an Ile–Val bond, also observed by Canfield [*J. Biol. Chem.*, **238**, 2691 (1963)], and the carboxymethylation of methionine.

and remains unexplained. The yields in Dowex chromatography of peptides MB and ME were, respectively, 80 and 95% (based upon sample applied to the column), demonstrating that tryptophan 108 was the only residue oxidized.[5]

The recent and elegant studies of Phillips, *et al.*,[6,7] have established the structure of the lysozyme molecule, and it is possible to relate aspects of the reactivity of tryptophan 108 to the crystallographic information. Blake[8] has shown that iodine specifically attacks tryptophan 108 in the crystal, which confirms the data for the soluble protein and justifies correlation of the solution chemistry with the crystallography. Solution studies show that the rate of reaction of iodine with tryptophan 108 is one-half that observed for this side chain in a model compound, N-acetyltryptophan ethyl ester. The reaction rates were measured using the disappearance of iodine color at 410 mμ, at pH 5.5, 0.1 *M* acetate. Second-order kinetics were followed to at least 40% of complete reaction. Unexpectedly, the basis of the high reactivity of tryptophan 108 cannot be accessibility to the reagent, as this residue is substantially less exposed than several other tryptophan side chains,[7] notably 62 and 63, which together with 108 are located in the active-site cleft. However, the

(5) The minor oxindole-containing peptide MG, found in only 1.4% yield, contained amino acids 98–108, and it is probably an artifact derived from ME.

(6) C. C. F. Blake, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc.* (London), in press.

(7) C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *ibid.*, in press.

(8) C. C. F. Blake, *ibid.*, in press.

crystallography[7] shows that the indole nitrogen of tryptophan 108 is hydrogen bonded to a main-chain carbonyl and the 2-carbon is in contact with the carboxyl of glutamic 35. Both interactions should increase the electron density of the ring and activate it with respect to attack by the iodine electrophile. Oxindole-108-lysozyme was inactive (less than 1% of full activity) against both cell walls and N-acetylglucosamine polymers, which might follow either from a disruption of substrate binding [binding of tri(N-acetylglucosamine) was not measurable after oxidation of tryptophan 108] or from a direct effect of the oxidation of tryptophan 108 upon one of the bond-cleaving groups, specifically the adjacent glutamic 35.[7] In contrast, oxindole-62-lysozyme, prepared according to Hayashi, *et al.*,[3] catalyzed the hydrolysis of penta(N-acetylglucosamine) at 7% the rate for the native enzyme, measured under conditions of substrate saturation. The presence of enzymic activity is in accord with the crystallography,[7] which shows that tryptophan 62 is distant from the cleavage site. The less-than-full activity observed for this derivative is not necessarily significant, owing to the complexity of the kinetics, to which both productive and nonproductive complexes contribute.[9] The assay for enzymic activity using cell walls is performed at substrate concentrations below saturation (J. A. Rupley, unpublished data) and, owing to the weaker binding of substrate to oxindole-62-lysozyme, it is understandable that lytic activity was not observed for the derivative.[3,10]

These experiments illustrate the excellent correlation between the chemistry and the crystallography in that the properties of tryptophans 62 and 108 can be predicted from the crystal structure. Although this in fact was realized because of the impetus supplied by the chemical studies, it appears, in contrast, highly implausible that one could accomplish the converse, namely, predicting from only their chemical properties the structure surrounding these residues, particularly tryptophan 108. As well as illustrating the complexity of the chemistry of proteins, the data underscore the difficulties inherent in the use of chemical modification to map an active site.

Acknowledgment. We are deeply indebted to Drs. Phillips, North, Blake, and their colleagues for the generosity they have shown in sharing and discussing the crystallographic information. This work was supported by Research Grant GB-3798 from the National Science Foundation.

(9) J. A. Rupley and V. Gates, *Proc. Roy. Soc.* (London), in press.
(10) Confirmed in this laboratory.
(11) To whom inquiries concerning this paper should be addressed.

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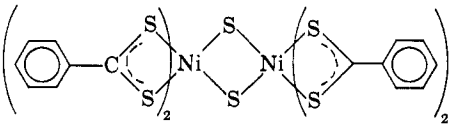
Further on the Myth of Nickel(IV)–Sulfur Chelates. V[1]

Sir:

From the studies of Holm and co-workers[2] and others[3] on the electrochemical oxidation of nickel(II) complexes of *o*-aminothiophenol and our work[1] on the addition of sulfur to 1,1-dithiols, it is apparent that the often quoted[4–6] work of Hieber and Brück[7] describing some sulfur-containing complexes as nickel(IV) species is incorrect. In this communication we present evidence which shows that the species formed by oxidation of nickel(II) dithiobenzoate and nickel(II) N-phenyldithiocarbamate do not contain nickel(IV) but are ligand-oxidized nickel(II) species.

The compounds we address ourselves to in this communication are the complexes I and II which have been formulated as nickel(IV) species.[4–7] They are



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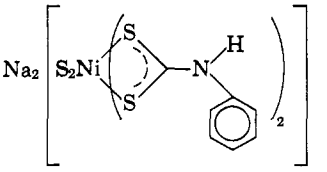
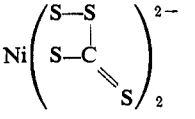


II

obtained by polysulfide or sulfur oxidation of the parent nickel(II) dithiobenzoate and nickel(II) dithiocarbamate complexes, respectively. Compound I is[7] a dark violet product reported to have a molecular weight in freezing benzene of 920 ± 150 (calculated 794). Compound II was not isolated in pure state by Hieber and Brück,[7] but the sodium salt was studied sufficiently well to evaluate its stoichiometry.

As part of our studies with 1,1-dithiol complexes, we have shown[1] that anionic complexes of nickel(II) of the type $Ni(S_2CS)_2^{2-}$, where X = $CHNO_2$, C(CN)-COC_6H_5, NCN, S, C(CN)$CONH_2$, and C(CN)$COOC_2$-H_5, are oxidized by iodine, sulfur, or polysulfides to products which are nickel(II) complexes of disulfide ligands. For example, the product formed by the oxidative addition of sulfur to $Ni(CS_3)_2^{2-}$ has been formulated as



based on its physical and chemical properties. These products are reduced by triphenylphosphine to the 1,1-dithiolate complex and $(C_6H_5)_3PS$.

(1) Part IV: D. Coucouvanis and J. P. Fackler, Jr., *J. Am. Chem. Soc.*, **89**, 1346 (1967).
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(3) E. J. Stiefel, J. H. Waters, E. Billig, and H. B. Gray, *ibid.*, **87**, 3016 (1965); L. F. Larkworthy, J. M. Murphy, and D. J. Phillips, *ibid.*, **88**, 1570 (1966).
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(5) J. C. Bailar, "Coordination Compounds," Monograph, No. 131, Reinhold Publishing Corp., New York, N. Y., 1956.
(6) F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," 1st ed, John Wiley and Sons, Inc., New York, N. Y., 1962, p 745.
(7) W. Hieber and R. Brück, *Z. Anorg. Allgem. Chem.*, **269**, 13 (1952).