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assay for enzymic activity using cell walls is performed at substrate concentrations below saturation (J. A. Rupley, unpublished data) and, owing to the weaker binding of substrate to oxindole-62-lysozyme, it is understandable that lytic activity was not observed for the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.

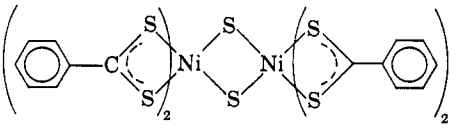
**F. J. Hartdegen, J. A. Rupley**[11]
*Department of Chemistry, University of Arizona*
*Tucson, Arizona*
*Received January 7, 1967*

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

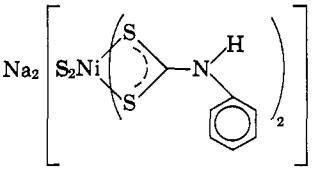
*Sir:*

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



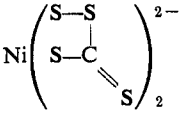
I



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 $(C_6H_5)_3PS$.

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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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(7) W. Hieber and R. Brück, *Z. Anorg. Allgem. Chem.*, **269**, 13 (1952).

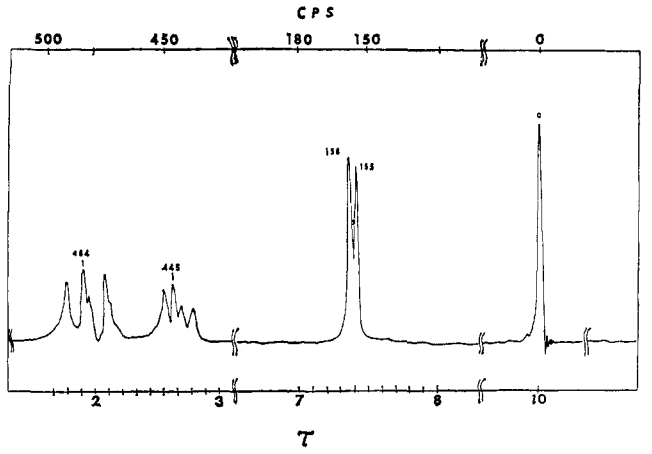
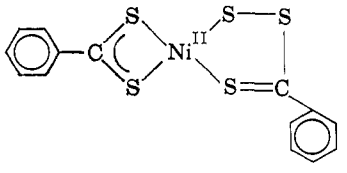



Figure 1. The spectrum of bis(*p*-dithiotoluato)nickel(II) in carbon disulfide at 60 Mc on a Varian A-60A nmr spectrometer.

We have prepared I in the following manner. By melting benzoic acid (11 g), $P_2S_5$ (40 g), and $(C_2H_5)_4NCl \cdot H_2O$ (18 g) together with stirring, an orange-brown mixture appears. Addition of $NiCl_2 \cdot 6H_2O$ (12 g) causes a vigorous evolution of HCl(g) and the formation of a violet-brown color. After cooling, I is extracted from the mixture with benzene, recrystallized from tetrahydrofuran–water, and then recrystallized from benzene. Violet crystals of I, mp 200–201° dec (lit.[7] mp 195° dec), are obtained. *Anal.* Calcd for $C_{14}H_{10}S_5Ni$: C, 42.3; H, 2.5; S, 40.0; Ni, 14.9; mol wt, 397. Found: C, 42.5; H, 2.6; S, 37.5; Ni, 15.0; mol wt (osmometrically), in $CHCl_3$, 350 ± 50; in $C_6H_6$, 447 ± 40, at $8 \times 10^{-3}$ *M*. A spectrophotometric titration of the complex with a standard solution of $(C_6H_5)_3P$ in $CHCl_3$ shows that one removable sulfur per nickel is present. Heating a $CHCl_3$ solution of the complex to 60° with a slight excess of $(C_6H_5)_3P$ present gives a quantitative yield of dithiobenzoate,[8] mp 221–22° (lit.[7] 219°). *Anal.* Calcd for $C_{14}H_{10}S_4Ni$: C, 46.0; H, 2.7; S, 35.1; Ni, 16.1. Found: C, 45.9; H, 2.8; S, 33.9; Ni, 16.0.

In a similar manner the "nickel(IV)" complex of the thio derivative of *p*-dithiotoluic acid is obtained, mp 219–221°. It also is reduced by $(C_6H_5)_3P$ to give nickel(II) *p*-dithiotoluate, mp 240–241°. All complexes were checked for purity by thin layer chromatography.

The ease with which I loses sulfur to $(C_6H_5)_3P$ suggested to us that I might be formulated as III by analogy with the sulfur-rich 1,1-dithiolate species.[1] The molecular weight in $CHCl_3$ and benzene at 37°

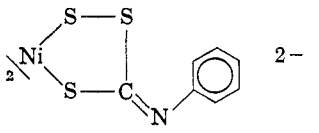


III

is consistent with this. The data at 6° in benzene of Hieber and Brück may indicate intermolecular association.[9] The mass spectrum of the complex (and the *p*-toluic acid derivative) shows no evidence in the vapor state of I but substantial indication of III.

The nuclear magnetic resonance spectrum (Figure 1) of the diamagnetic *p*-toluic acid derivative also strongly supports the formulation of the sulfur addition product as III rather than I. The species shows two distinct $-CH_3$ resonances separated by ~2.7 cps in carbon disulfide. This is consistent with the presence of two electronically different $-CH_3$ groups.[10]

The complex formulated by Hieber and Brück as II appears to be the sulfur-rich dithiocarbimate IV, a species similar to the sulfur-rich N-cyanodithiocarbimate we have reported.[1] By heating bis(N-



IV

phenyldithiocarbamate)nickel(II) with sodium hydride in tetrahydrofuran, the N–H stretch disappears, and one can isolate the dianionic N-phenyldithiocarbimate complex as the tetra-*n*-butylammonium salt. It is a 2:1 electrolyte in nitromethane and behaves as a typical square-planar, 1,1-dithiolate[11] complex of nickel(II). It adds sulfur in a manner similar to that which we have already reported[1] for other 1,1-dithiolates. Hieber and Brück obtained their "nickel(IV)" species by polysulfide oxidation of a very basic solution containing the dithiocarbamate complex. Such a solution certainly would contain the anionic dithiocarbimate complex. It is interesting to note that Hieber and Brück failed to form a "nickel(IV)" species with nickel(II) diethyldithiocarbamate, a species which cannot form a stable dithiocarbimate.[12]

While there remain some complexes[13] which may contain nickel(IV) such as the hydrous oxides and $K_2NiF_6$, the number of well-authenticated examples is very small. Indeed, the degree to which ligand oxidation better describes the resulting complex remains to be determined, especially with ligands such as oximes, imines, and *o*-bis(dimethylarsine)benzene. It is already apparent that neutral 1,2-dithiol complexes of nickel, formed by oxidation of the planar dianionic

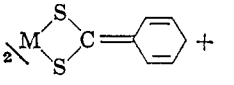
(8) Sulfur addition (catalyzed by $Ph_3P$) to the blue nickel(II) dithiobenzoate in dioxane produces a violet solution having a spectrum identical with that of the sulfur-rich species.

(9) It is well known that intermolecular association can occur with sulfur–ligand complexes. However, the limited solubility in benzene leads us to question the accuracy of a cryoscopic molecular weight measurement.

(10) The Hieber–Brück species would be expected to have two geometrical isomers with electronically similar dithiobenzoate ligands.

(11) J. P. Fackler, Jr., and D. Coucouvanis, *J. Am. Chem. Soc.*, 88, 3913 (1966).

(12) It is not entirely clear why only one sulfur oxidatively adds to nickel(II) dithiobenzoate. However, it does appear that some resonance delocalization is lost on sulfur addition if our formulation is correct. Hence a second sulfur atom addition may not be energetically feasible. The nickel(II) dithiobenzoate complex itself possibly adds sulfur only because of substantial contributions of resonance structures of the type



to the description of the electronic structure of the complex.

(13) F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," 2nd ed, Interscience Publishers, Inc., New York, N. Y., 1966 pp 891, 892.

nickel(II) species, should not be described as nickel(IV) complexes.[2,3,14,15]

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**J. P. Fackler, Jr., D. Coucouvanis**
*Department of Chemistry, Case Institute of Technology*
*Cleveland, Ohio 44106*
*Received November 30, 1966*

## Alkylation of Chymotrypsin by α-Bromo-4-nitroacetophenone, a Charge-Transfer Acceptor

*Sir:*

We have observed that a new long-wavelength absorption band is formed when chymotrypsin (ChT)[1] is alkylated with α-bromo-4-nitroacetophenone (BrNAP).[1] In this communication, we present evidence that this relatively intense absorption band ($\lambda_{max}$ 350 mμ ($\epsilon$ 7.55 × $10^3$ $M^{-1}$ $cm^{-1}$)), which is not shown by either enzyme or the alkylating agent alone, is a "charge-transfer band" arising from the interaction of a tryptophan residue of the enzyme and the 4-nitroacetophenone moiety.

BrNAP reacts with ChT at methionine-192 (*vide infra*) to yield a monoalkylated derivative which is inactive toward N-acetyl-L-tyrosine ethyl ester. Evidence for the 1:1 stoichiometry is obtained from amino acid analysis and the absorption spectrum of ChT–NAP in the 260–290-mμ region. At these wavelengths, the absorption spectrum of ChT–NAP is essentially the sum of the spectra of ChT and BrNAP. The rapid rate of inactivation of ChT by BrNAP is closely paralleled by the rate of increase of the new absorption band at 350 mμ.

Amino acid analysis of ChT–NAP showed that neither of the two histidines of ChT had been modified. On the other hand, the methionine content of the alkylated enzyme was low (1.5 Met/mole) compared to that of the native enzyme (2.0 Met/mole). In order to prove that a single methionine had been alkylated, both ChT and ChT–NAP were oxidized with performic acid according to the method of Hirs.[2] Amino acid analysis of performic acid oxidized ChT (following acid hydrolysis) indicated that both methionines had been converted to methionine sulfone; no methionine could be detected. In contrast, similar treatment of performic acid oxidized ChT–NAP yielded only one methionine sulfone/mole; and, in addition, 0.5 methionine/mole of enzyme was found. Since alkylated methionines are stable to performic acid oxidation,[3] but are only partially regenerated to methionine on acid hydrolysis,[4] the above results show that BrNAP alkylates one of the enzyme's two methionines.

In order to determine which of the two methionines (192 or 180) had been alkylated, the enzyme and the alkylated enzyme were treated with hydrogen peroxide under conditions which convert methionine-192 to methionine sulfoxide.[5,6] Whereas $H_2O_2$ treatment of native ChT, followed by basic hydrolysis,[3] yielded equimolar amounts of methionine and methionine sulfoxide, identical treatment of ChT–NAP yielded no methionine sulfoxide and the same amount of methionine as ChT–NAP that was not treated with $H_2O_2$. These experiments show that methionine-192 of ChT–NAP is protected against oxidation by $H_2O_2$. Therefore, the methionine alkylated by BrNAP is methionine-192.

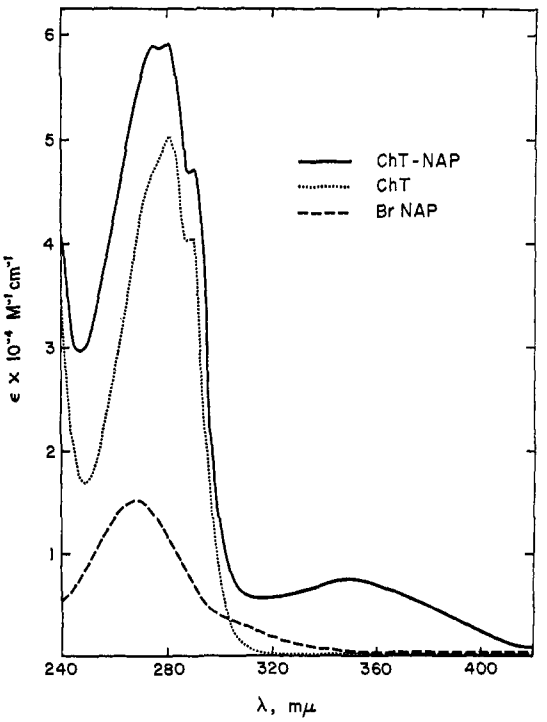


Figure 1. Absorption spectra of chymotrypsin (ChT), chymotrypsin alkylated with α-bromo-4-nitroacetophenone (ChT–NAP), and α-bromo-4-nitroacetophenone (BrNAP) at 25° in 0.001 *M* HCl. The spectrum of BrNAP was obtained in a solution containing 5% acetonitrile.

The assignment of the new long-wavelength absorption band[7] of ChT–NAP (Figure 1) as a charge-transfer transition is supported by the following evidence. (a) Alkylation of chymotrypsin with α-bromo-2,4-dinitroacetophenone yields a modified enzyme possessing a new absorption band with $\lambda_{max}$ 365 mμ. The red shift in $\lambda_{max}$ (from 350 to 365 mμ) upon increasing the electron affinity of the acceptor (nitroacetophenone to dinitroacetophenone) is consistent with the assignment of the new absorption band as a charge-transfer transition.[8] (b) When ChT–NAP and ChT–DNAP are heated to 65°, they completely lose their characteristic long-wavelength transitions. Upon cooling the heat-denatured alkylated proteins, the long-wavelength absorption bands reappear. (c) The new absorption band of ChT–NAP can also be destroyed

(1) The abbreviations used are ChT, chymotrypsin; BrNAP, α-bromo-4-nitroacetophenone; ChT–NAP, chymotrypsin alkylated with BrNAP; and ChT–DNAP, chymotrypsin alkylated with α-bromo-2,4-dinitroacetophenone.
(2) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).
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(4) H. G. Gundlach, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **234**, 1761 (1959).
(5) H. Weiner, C. W. Batt, and D. E. Koshland, Jr., *ibid.*, **241**, 2687 (1966).
(6) H. Schachter and G. H. Dixon, *ibid.*, **239**, 813 (1964).
(7) This absorption band is optically active. It has a molecular ellipticity of 2.56 × $10^4$ deg $cm^2$ $dmole^{-1}$ at 350 mμ.
(8) E. M. Kosower, "Molecular Biochemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 183.