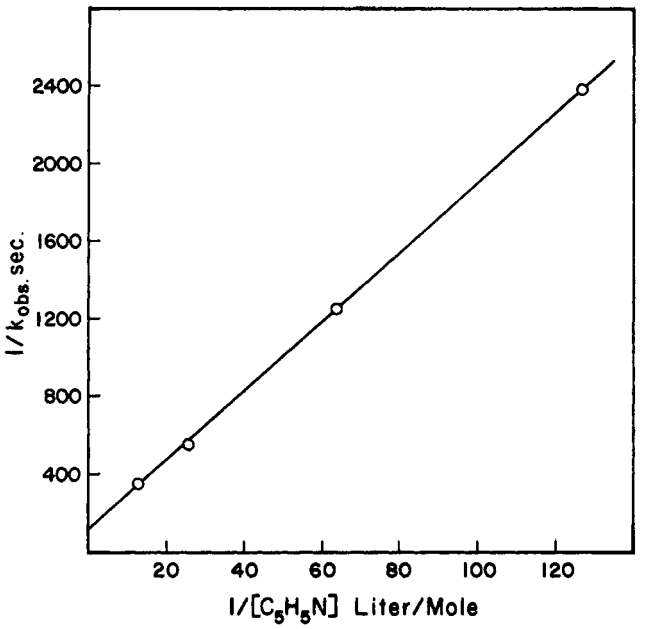
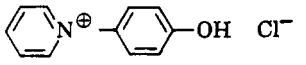


Fig. 1.—Photo-induced reaction of *p*-nitrophenyl phosphate with pyridine in aqueous solution.

and ultraviolet (λ_{max} of II, 297 and 267 mμ; λ_{max} of I, 310 mμ) spectral data. The structure of II was confirmed by hydrolysis in concentrated hydrochloric acid at 100° to phosphoric acid and 1-(*p*-hydroxyphenyl)-pyridinium chloride (III). The latter, isolated in 50% yield, was identical in melting point, infrared and ultraviolet spectra, chromatographic behavior, and properties of the picrate derivative with an authentic sample of N-(*p*-hydroxyphenyl)pyridinium chloride prepared from *p*-aminophenol.[5]



III

In contrast to the results of the photochemical reaction, *p*-nitrophenyl phosphate was converted to *p*-nitrophenol when heated with an aqueous pyridine solution.

Rate data for the photo-induced reactions of *p*- and *m*-nitrophenyl phosphate are presented in Table I.

TABLE I

PHOTO-INDUCED REACTIONS OF *p*- AND *m*-NITROPHENYL PHOSPHATE IN AQUEOUS PYRIDINE SOLUTIONS[a]

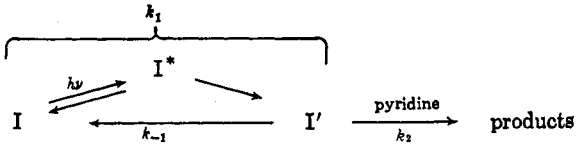
Isomer	$10^2 \times$ [pyridine], moles/l.	$10^4 \times k_{obsd}$,[b] sec.$^{-1}$ Ester disappearance	Nitrophenol appearance
para	0	(0.017)	(0.005)
	0.79	0.420	0
	1.58	0.802	
	3.95	1.82	
	7.90	2.89	
meta	0	0.648	0.588
	0.79	0.693	0.573
	7.90		0.635

[a] Temperature +3°, *p*-nitrophenyl phosphate concentration 1.00×10^{-4} *M*, *m*-nitrophenyl phosphate concentration 1.33×10^{-4} *M*, pH 9.8 (0.004 *M* borate buffer). [b] In determining k_{obsd} experimental infinity values were used except for reaction of the *para* isomer in absence of pyridine, in which case it was assumed that the absorbance of the product was the same as that for nitrophenol.

(5) Prepared by the method of N. E. Grigor'eva and M. D. Yavlinskii, *Ukr. Khim. Zh.*, **18**, 82 (1952); *Chem. Abstr.*, **48**, 11411a (1954).

Ester disappearance was followed by the absorbance decrease at 310 mμ for the *para* isomer and at 275 mμ for the *meta* isomer; nitrophenol appearance was followed by the absorbance increase at 400 mμ. First-order kinetics were obtained for each individual run. It may be noted that (a) the observed first-order rate constants for disappearance of *p*-nitrophenyl phosphate depended upon the pyridine concentration, being approximately proportional to the pyridine concentration for the more dilute solutions, (b) *p*-nitrophenol was not formed in these reactions, and (c) the over-all rate of reaction of *m*-nitrophenyl phosphate was little affected by pyridine, whether observed at 275 or 400 mμ.

These data are consistent with the view that photo-excited *p*-nitrophenyl phosphate (I*) yields an intermediate (I′) which may either return to *p*-nitrophenyl phosphate in the ground state or undergo displacement by pyridine in a second-order process. On the basis

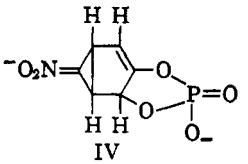


of this mechanism and the steady-state approximation, the following relation holds

$$\frac{1}{k_{obsd}} = \frac{k_{-1}}{k_1 k_2 [\text{pyridine}]} + \frac{1}{k_1}$$

As shown in Fig. 1, the rate data fit this equation well. From the intercept and slope of the line it is found that $k_{-1}/k_2 = 0.14$ mole/l.

Possibilities for I′ include a triplet state or a cyclic phosphate (IV).[6] The nature of the intermediate and the generality of photo-induced aromatic substitution reactions are under investigation.



IV

(6) For a discussion of the photo-excited state, I*, of *p*-nitrophenyl phosphate see H. E. Zimmerman and S. Somasekhara [*J. Am. Chem. Soc.*, **85**, 922 (1963)].

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RECEIVED FEBRUARY 8, 1964

An Environmentally-Sensitive Reagent with Selectivity for the Tryptophan Residue in Proteins[1]

Sir:

In order to explore the properties of enzymes during their catalytic action, we have been interested in compounds which would be sensitive to changes in environment and which could be introduced at specific positions in the protein molecule. The first such com-

(1) Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

pound that has been found to be useful is 2-hydroxy-5-nitrobenzyl bromide (I).

To synthesize I, the chloromethylation procedure of Buchler, *et al.*,[2] was followed, except that HBr was substituted for HCl. A crystalline material was isolated, recrystallized from benzene, and was found to melt sharply at 145°. Elemental analysis showed: C, 36.52; H, 2.46; Br, 34.37. Calcd. for $C_7H_6BrNO_3$: C, 36.23; H, 2.61; Br, 34.44.

In a typical experiment, 8 μmoles of α-chymotrypsin in 20 ml. of an 8 *M* urea–5% methanol solution at pH 3 was treated with 80 μmoles of solid I. After 0.5 hr., the chymotrypsin was separated from excess reagent by gel filtration on Sephadex G-25 which had been pre-equilibrated with 8 *M* urea at pH 3. Urea was removed from the protein fraction using Sephadex G-25 pre-equilibrated with 0.001 *N* HCl. The preparation was dialyzed against 0.001 *N* HCl to remove the last traces of impurity and the protein sample was then lyophilized. Assuming that the extinction coefficient of the protein is unchanged by the reaction and that the chromophoric group on the protein has the same extinction coefficient (18,900) as 2-hydroxy-5-nitrobenzyl alcohol, approximately 1 mole of the benzyl derivative is covalently bonded per mole of protein. Amino acid analysis by the method of Spackman, *et al.*,[3] after basic or acid hydrolysis showed no changes in any residue except tryptophan. The tryptophan content had decreased by 1 residue (*cf.* Table I).

TABLE I

AMINO ACID MODIFICATION ON TREATMENT OF CHYMOTRYPSIN WITH 2-HYDROXY-5-NITROBENZYL BROMIDE

Expt. no.	Experimental conditions	No. of 2-OH-4-NO_2-benzyl groups per enzyme molecule (calcd. from spectra)	Amino acid residues found per molecule of enzyme[a]				
			Met	His	Try	Lys	Tyr
1	4×10^{-4} *M* enzyme, 4×10^{-3} *M* compd. I, 8 *M* urea	1.0	1.94		5.94	13.2	3.9
2	4×10^{-4} *M* enzyme, 2×10^{-2} *M* compd. I, 8 *M* urea	4.7	1.8	1.9	2.2	13.3	3.8
3	Control	None	1.9	1.96	6.87	13.1	3.8

[a] No detectable changes in other amino acid residues. Values corrected for yield by assuming 6.0 residues of phenylalanine.

Repetition of the experiment with five times the concentration of I caused the introduction of 4.7 chromophoric groups per molecule of enzyme (*cf.* Table I). When the experiment was performed in the absence of urea, from 1 to 3 residues of label were introduced depending on experimental conditions. For example, at pH 3, with 4×10^{-5} *M* enzyme and 4×10^{-3} *M* compound I, 2.3 residues of chromophoric group were introduced per molecule of enzyme.

A further check on the selectivity was obtained by reaction of the 2-hydroxy-5-nitrobenzyl bromide with an aqueous solution of those amino acids present in α-chymotrypsin, *i.e.* all the usual ones except cysteine. An amino acid mixture (each amino acid = 0.01 *M*) and 0.03 *M* in compound I was mixed at pH 3. After reaction was complete, an aliquot was analyzed without further treatment. All amino acids were unchanged except tryptophan which had reacted completely. An interesting feature of this reagent is the lack of formation of a methionine derivative. In separate experiments, benzyl bromide was found to react with methionine as well as tryptophan and sulfhydryl groups. By analogy to other alkylating agents, the reagent would be expected to react with cysteine, and preliminary results with the free amino acids indicate that this is so. However, the relative reactivity of cysteine residue compared to tryptophan in proteins needs careful study in a variety of proteins. At present, it can be said that the reagent shows specificity for tryptophan in reactions performed in acidic solutions with proteins lacking the SH group.

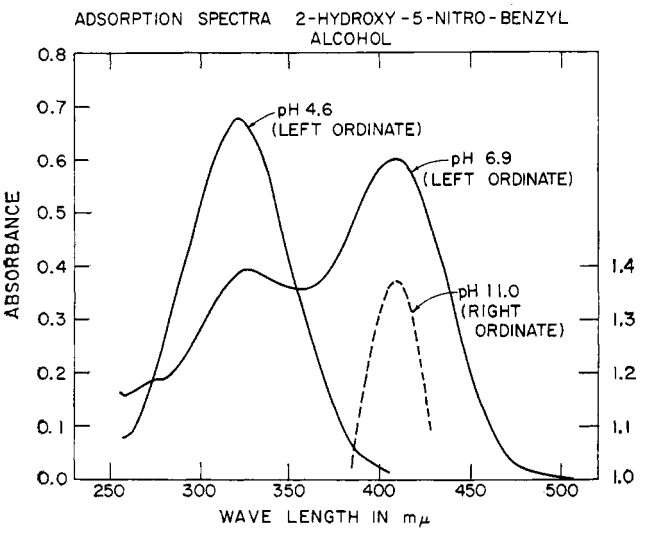

Fig. 1.—Absorption spectra of 2-hydroxy-5-nitrobenzyl alcohol at various pH values.

Several features of this reagent are worthy of note. The first is its selectivity which makes it of particular value in modification studies. In proteins which do not contain free SH groups, the reagent is specific for tryptophan. Since it is possible to mask SH groups reversibly, *e.g.*, as Stracher has done for myosin,[4] even in proteins containing SH groups selective tryptophan reactivity can be obtained. Other reagents which react with tryptophan, such as N-bromsuccinimide,[5] and photooxidation[6] are useful but are not specific. Hence, this reagent will aid in ascertaining the role of tryptophan in proteins. The second feature of the reagent is its chromophoric group which is sensitive to changes in environment and absorbs in a region of the spectrum in which the protein itself is transparent (*cf.* Fig. 1). The p*K* of the phenolic group in this compound is about 7. The acid form absorbs in the 320 mμ region and the basic form absorbs in the 410 mμ region. Thus, the reagent is sensitive to changes in pH and to environmental changes which can be measured in both the 320 mμ and the 410 mμ absorption regions. Finally, it appears that the extinction coefficient of the protein derivative is sufficiently predictable to allow a calculation of the number of residues

(2) C. A. Buchler, F. R. Kirchner, and G. F. Deebel, *Org. Syn.*, **20**, 59 (1940).

(3) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(4) A. Stracher, *Biochem. Biophys. Res. Commun.*, **13**, 279 (1963).

(5) T. Viswanatha and W. B. Lawson, *Arch. Biochem. Biophys.*, **93**, 128 (1961); T. Viswanatha, W. B. Lawson, and B. Witkop, *Biochim. Biophys. Acta*, **40**, 216 (1960).

(6) L. Weil, S. James, and A. R. Buchert, *Arch. Biochem. Biophys.*, **46**, 266 (1953); W. J. Ray, Jr., and D. E. Koshland, Jr., *J. Biol. Chem.*, **237**, 2493 (1962).

absorbed without laborious amino acid analyses, a feature which has virtue in studies of amino acid residue reactivities.

It is clear that the general principles allow various permutations. Thus, compounds can be made having other spectral properties, *e.g.*, compounds that absorb in other regions of the spectrum or that fluoresce. Similarly, variation of the reactive group may produce compounds which are specific for other residues, *e.g.*, tyrosine. The application of this and similar reagents will be reported in subsequent publications.

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RECEIVED JANUARY 4, 1964

Effect of Distant Atoms in a Molecule on Nuclear Magnetic Resonance Chemical Shifts

Sir:

Proton nuclear magnetic resonance (n.m.r.) spectra of methyl groups terminating long chains of atoms have been found to be unusually sensitive to structural changes far distant[1] from the methyl group in the molecule, when sulfur or selenium is incorporated in the molecular backbone. For example, careful assignment of proton nuclear magnetic resonances in the product obtained by reaction of dimethyl sulfate with sulfur trioxide shows that separate resonance peaks can be observed for n as great as 6 in the following molecule

$$CH_3O\left[\begin{matrix}O\\SO\\O\end{matrix}\right]_nCH_3$$

As n increases, the difference in chemical shift, $\Delta\delta$, diminishes from 0.23 p.p.m. for molecules having values of $n = 1$ and $n = 2$ to the nearly indistinguishable difference of 0.005 p.p.m. for the molecules with $n = 5$ and $n = 6$.

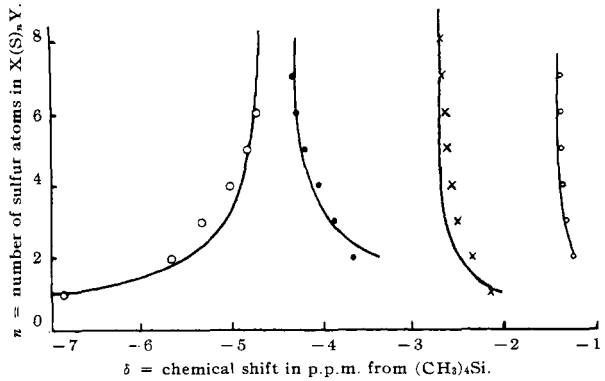

Fig. 1.—Proton n.m.r. chemical shifts of the CH_2 or CH_3 hydrogen atoms in the substituent X of polysulfide molecules of the type $X(S)_nY$ as a function of the chain length: O, X = CH_3 and Y = Cl; ●, X = Y = $CH_2(C_6H_5)$; ×, X = Y = CH_3; ○, X = Y = $C(CH_3)_3$.

Similar long-range effects are observed with sulfur chains having the structure $X(S)_nY$ and selenium chains having the structure $X(Se)_nX$, where the proton resonance of the X group is observed and X has been chosen to be CH_3, $C(CH_3)_3$, or $CH_2(C_6H_5)$. Typical data for four different polysulfide reaction mixtures[2] are shown in Fig. 1.

These data may be fitted to an approximation based on the theory for the so-called "neighbor anisotropy effect."[3] If it is assumed that the local magnetic susceptibility tensor on the distant group or atom is axially symmetric, one has the following equation for its contribution $\Delta\sigma$ to the magnetic screening constant, σ, of the nucleus being observed

$$\Delta\sigma = [(\Delta\chi/3N_0)\langle 1 - 3\cos^2\gamma\rangle_{av.}]/R^3 \quad (1)$$

where $\Delta\chi/3N_0$ is a constant, R is the distance between the center of the contributing group and the nucleus in question, and γ is the angle between the axis of anisotropy of this group and the vector R. In order to avoid problems with bulk magnetic susceptibility and other contributions to the over-all screening constant, we have treated our data in terms of the difference in chemical shift between molecules having $(n + 2)$ units in the chain and those of infinite length, assuming that eq. 1 can be reduced to the form C/R^3, where C is a constant. Thus, for the molecule $X(Z)_nY$, we have the following equation for the difference in chemical shift, $\Delta\delta$, in which C_y is the shift contribution of the terminal group Y separated from the group X, containing the magnetic nucleus, by n Z atoms or moieties and C_z is the shift contribution of each Z

$$\Delta\delta = \delta_n - \delta_\infty = C_y/R_{n+1}^3 + C_z\left(\sum_{j=1}^{n} 1/R_j^3 - \sum_{j=1}^{\infty} 1/R_j^3\right) \quad (2)$$

A reasonable *a priori* assumption for estimating the value of R is to use the random-flight calculation of the end-to-end distance for a chain having free rotation at tetrahedral bond angles.[4]

$$R = j - \sum_{k=1}^{j-1} 2(j-k)/3^k \quad (3)$$

where

$$\sum_{j=1}^{\infty} 1/R_j^3 = 1.750 \quad (4)$$

Application of eq. 2 and 3 to the data of Fig. 1 gives curves of the correct general shape. The lines in Fig. 1 were calculated on the basis of $C_y = +9$ for X = Y = CH_3, -17 for X = CH_3 and Y = Cl, $+30$ for X = Y = $CH_2(C_6H_5)$, $+9$ for X = Y = $C(CH_3)_3$, and $+18$ for S in the molecule $X(S)_nY$. The fit between the data and the theoretical curves is reasonably good but far from perfect. It can be improved by allowing the value of the shift constant for sulfur to be somewhat dependent on the end groups in the $X(S)_nY$ molecules, thereby allowing in part for chain-

(1) The major applications of "seeing-in-depth" within molecules by n.m.r. have been in the sequence distribution of polymers [references given in *Chem. Eng. News*, **41**, No. 16, 36 (1963)] and the interpretation of molecular-size distributions in novel families of compounds [K. Moedritzer and J. R. Van Wazer, *J. Am. Chem. Soc.*, **86**, 802, 807, 814 (1964)]. In most cases, n.m.r. chemical shifts are affected by atoms no more than four or five positions removed in the structure. For this effect in C^{13} spectra, see E. G. Paul and D. M. Grant, *ibid.*, **85**, 1701 (1963), who show that their additive shift parameters become constant for butyl and longer n-alkyl groups.

(2) Proof of the existence and structure of the new compounds reported in Fig. 1 (particularly the series $CH_3(S)_nCl$) is presented in a forthcoming paper.

(3) H. M. McConnell, *J. Chem. Phys.*, **27**, 226 (1957); J. A. Pople, *Proc. Roy. Soc.* (London), **A239**, 550 (1957).

(4) H. Eyring, *Phys. Rev.*, **39**, 746 (1932); F. T. Wall, *J. Chem. Phys.*, **11**, 67 (1943); see also P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 414.