chromatography (compare section A (b): white powder, infrared spectrum nearly identical with β-dextrin, no phenyl absorption at 1510 cm.$^{-1}$.

Anal. Calcd. for $C_{42}H_{69}O_{35} \cdot PO_3Na_2$ (mol. wt., 1258): C, 40.0; H, 5.62; P, 2.48. Found: C, 39, 39; H, 4.92; P, 2.36.

(b) *Quantitative Determination of the Reaction Products.* A standard run with β-dextrin was stopped after 24 hr. (32% cleavage) and the reaction products were determined by the following *phenol* method. Standard solutions were prepared by diluting 10^{-3} *M* phenol solution 2-fold, 5-fold, and 20-fold. Diazotized sulfanilic acid (0.1%) was added and the color determined photometrically (425 mμ, Perkin-Elmer 4000). The standard curve thus obtained was used. A 32% cleavage of pyrophosphate gave 29% phenol. A run

without β-dextrin (interrupted after 20% cleavage) showed 1.5% phenol.

(c) *Determination of Diphenyl Pyrophosphate and Monophenyl Phosphate from the Chromatogram. Determination of the Proportion of Diphenyl Pyrophosphate to Monophenyl Phosphate on Cleavage.* Ultraviolet absorption of diphenyl pyrophosphate showed λ_{max} 260 mμ (ϵ 8.75 $\times$ 10^2); monophenyl phosphate showed λ_{max} 266 mμ (ϵ 6.75 $\times$ 10^2). The chromatograms of the run (6.25 $\times$ 10^{-4} *M*) were eluted quantitatively according to section A(b). The solutions of the eluate would be on complete cleavage 2 $\times$ 6.25 $\times$ 10^{-4} mole of monophenyl phosphate (O.D. = 1.68, *d* 2.0 relating to 100%); without cleavage, 6.25 $\times$ 10^{-4} mole of diphenyl pyrophosphate (O.D. = 0.546, *d* = 1.0, relating to 100%). The values found are shown in Table VII.

A Highly Reactive Colored Reagent with Selectivity for the Tryptophan Residue in Proteins. 2-Hydroxy-5-nitrobenzyl Bromide[1]

H. Robert Horton and D. E. Koshland, Jr.

Contribution from the Biology Department, Brookhaven National Laboratory, Upton, New York. Received November 6, 1964

The environmentally sensitive protein reagent, 2-hydroxy-5-nitrobenzyl bromide, was shown to react rapidly with tryptophan in aqueous solutions over a wide range of pH. In acidic or neutral solutions it was highly specific in its reaction; the only other amino acid which was modified by the reagent was cysteine, whose reactivity was no more than one-fifth that of tryptophan. In alkali, tyrosine and cysteine were found to react to almost the same extent as tryptophan. Methionine did not form a stable derivative with the reagent in aqueous media, although it did catalyze the tryptophan reaction. The high order of reactivity of 2-hydroxy-5-nitrobenzyl bromide and its specificity for tryptophan are properties which contrast with those of 2-methoxy-5-nitrobenzyl bromide or unsubstituted benzyl bromide, and apparently involve the participation of the o-hydroxyl substituent in activation of the reagent. Environmental sensitivity of its absorption spectrum, ease of reaction with amino acids under mild conditions, and specificity for tryptophan contribute to the usefulness of 2-hydroxy-5-nitrobenzyl bromide as a protein-modification reagent.

One of the most important tools in the correlation of protein structure with function is the protein reagent. In general, the more specific the reagent the more valuable it is, although "broad spectrum" reagents are useful also. For the delineation of the role of tryptophan a number of reagents which fit in the latter category are known. Weil and co-workers have used photooxidation which modifies tryptophan, histidine, methionine, cysteine, and tyrosine.[2] Witkop and co-

workers[3] have described the valuable reagent N-bromosuccinimide which modifies tryptophan, histidine, tyrosine, SH, and probably methionine.[4] Iodination under appropriate circumstances modifies tryptophan and also tyrosine, methionine, and cysteine.[5,6]

In a previous publication the synthesis and reactivity of a new reagent, 2-hydroxy-5-nitrobenzyl bromide, hereafter designated as ϕ' Br,[7] were described.[8] It was found that in acid and neutral solutions this reagent reacted readily with tryptophan residues of chymotrypsin, and did not react with any other of the amino acids. The reagent absorbs in a region of the spectrum in which the protein is transparent and its spectral absorption is sensitive to environment. Although the reagent was not absolutely specific (it showed a sluggish

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

(2) L. Weil, S. James, and A. R. Buchert, *Arch. Biochem. Biophys.,* **46,** 266 (1953).

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

(4) Although it has not been reported that methionine is oxidized by N-bromosuccinimide, it is known that most oxidizing agents, including ICl, do oxidize this residue. Since it has been demonstrated [W. J. Ray, Jr., and D. E. Koshland, Jr., *J. Biol. Chem.,* **237,** 2493 (1962)] that methionine is regenerated from methionine sulfoxide during acid hydrolysis, it seems probable that the oxidation of this residue has not been observed in the past because of the acid treatments prior to analysis.

(5) L. K. Ramachandran, *Chem. Rev.,* **56,** 199 (1956).

(6) M. E. Koshland, F. M. Englberger, M. J. Erwin, and S. M. Gaddone, *J. Biol. Chem.,* **238,** 1343 (1963).

(7) Abbreviations: ϕ' Br, 2-hydroxy-5-nitrobenzyl bromide; ϕ'OH, 2-hydroxy-5-nitrobenzyl alcohol; ϕ'-Try, 2-hydroxy-5-nitrobenzyl derivative of tryptophan, precise structure unknown; ϕ'-Cys, 2-hydroxy-5-nitrobenzyl derivative of cysteine.

(8) D. E. Koshland, Jr., Y. D. Karkhanis, and H. G. Latham, *J. Am. Chem. Soc.,* **86,** 1448 (1964). By raising the temperature of bromomethylation it has been possible to obtain the bisbromomethyl derivative (m.p. 155–156.5). Control of the temperature at 70° is, therefore, desirable when high yields of the monobromomethyl derivative are desired.

reactivity with cysteine), it was far more specific than existing reagents and appeared to be specific for tryptophan in proteins such as chymotrypsin which lack sulfhydryl groups. Because of the potential usefulness of the reagent and because of its unusual reactivity, further studies on its properties were carried out and are reported here.

Experimental

The reagent 2-hydroxy-5-nitrobenzyl bromide (ϕ' Br) was synthesized by bromomethylation of p-nitrophenol at 70° as previously indicated,[8] and was recrystallized twice from warm benzene. The solutions of L-amino acids employed in this study were prepared from A grade compounds purchased from California Corp. for Biochemical Research, and from Type 1 Amino Acid Calibration Mixtures obtained from Beckman Instruments, Inc. Amino acid analyses were performed using the automatic apparatus of Spackman, et al.,[9] employing a somewhat larger than normal column (0.9 × 20 cm.) for quantitative determinations of tryptophan. Chromatographic columns were prepared from Beckman types 15A and 150A sulfonated styrene–divinylbenzene copolymer resins. Ribonuclease A was obtained from Worthington Biochem. Corp.

Unless specified otherwise, aqueous solutions of amino acids or proteins at room temperature at the indicated pH values were allowed to react in the dark with a solution of 2-hydroxy-5-nitrobenzyl bromide dissolved in dried acetone. The pH was maintained during reaction by addition of small increments of 1 N NaOH using a pH-stat to maintain the pH. A 5% acetone concentration was chosen because the proteins under study were not denatured at this concentration and it permitted concentrations of ϕ' Br up to 0.01 M. Absorption spectra were recorded with a Cary Model 14 spectrophotometer.

Results

Reaction of ϕ' Br with Amino Acids. Aqueous solutions of amino acids were treated with the reagent under a variety of conditions. Results of a typical experiment in terms of amino acid recovery data are presented in Table I. In this experiment a solution which was 0.001 M in each amino acid (except cystine) was treated at pH 3.0 ± 0.1 with 0.01 M ϕ' Br. The experiment was conducted under an atmosphere of nitrogen.

This solution could be placed directly on the amino acid analyzer for analyses of all amino acids except cysteine. It was found, however, that the cysteine content was low under these conditions and that cysteine peaks appeared in a number of positions on the chromatogram. Since the buffer and ninhydrin systems contain reducing agents which prevent oxidation of cysteine, and the recovery of cysteine is deduced from the cystine peak on the analyzer, it was clear that some variation was needed to obtain reliable values. The procedure which was found to be successful involved oxygenation at pH 7.5 after reaction with ϕ' Br and prior to addition of the sample to the analyzer. When this oxygenation step was used, the amount of cystine in a control experiment was in good agreement with the amount of cysteine plus cystine added initially.

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

Table I. Reaction of Amino Acids with 2-Hydroxy-5-nitrobenzyl Bromide (ϕ' Br) at pH 3[a]

	Recovery (μmoles)		
Amino acid	Control, not oxygenated	Control, oxygenated	ϕ' Br-treated, oxygenated
Tryptophan	1.00	1.00	0.00
Lysine	1.00	1.00	1.00
Histidine	1.00	1.00	(1.09)[b]
Arginine	1.02	1.02	1.02
Aspartic acid	1.00	1.005	1.01
Threonine	1.00	1.00	1.00
Serine	1.005	0.99	1.00
Glutamic acid	1.00	1.00	1.00
Proline	(1.18)[b]	0.996	0.99
Glycine	1.00	1.005	1.01
Alanine	1.00	1.01	1.01
Half-cystine	(1.12)[c]	(1.94)[c]	(1.74)[c]
Valine	1.00	1.01	1.02
Methionine	1.00	0.98	0.93
Meth. sulfoxides	0.01	0.03	0.07
Isoleucine	0.99	1.00	1.00
Leucine	0.99	1.00	0.99
Tyrosine	1.00	0.98	0.99
Phenylalanine	1.00	0.99	0.99

[a] Conditions: Each amino acid, 10^{-3} M; 0.01 M ϕ' Br added as 0.2 M solution in acetone; pH 3.0 ± 0.1; final acetone concentration 5% (v./v.). Sample designated "control, not oxygenated" was placed on analyzer directly after adjustment of pH and volume; "control, oxygenated" and "ϕ' Br-treated, oxygenated" samples were placed in an atmosphere of oxygen at pH 7.5 for 24 hr. before readjustment of pH and volume for application to analyzer. [b] Independent experiments indicate other components eluted with the designated amino acid as described in text. For example, one of the cysteine products appears under proline in the nonoxygenated control. [c] Sample was 0.0005 M in cystine and 0.00095 M in cysteine initially.

Examination of the results shown in the third column of Table I reveals that upon treatment with 0.01 M ϕ' Br: (a) tryptophan has completely disappeared from solution; (b) histidine appears slightly elevated; (c) cysteine has reacted to an extent of approximately 20% (i.e., 0.74 of the 0.94 μmole represented by this aliquot is recovered as cystine after oxygenation); and (d) none of the other amino acids' recoveries are altered by this treatment within the limits of detection. The amount of methionine sulfoxides varied slightly with individual experiments, but in all cases the methionine sulfoxides plus unchanged methionine accounted for the total methionine initially added.

In separate experiments in which solutions containing 0.001 M histidine were treated with 0.01 M ϕ' Br in the absence of cysteine, no change in histidine recovery could be detected. Moreover, treatment of cysteine with ϕ' Br in the absence of other amino acids produced a derivative which is eluted in the histidine portion of the 15–20-cm. column as shown in Figure 1. The unchanged histidine plus the cysteine derivative of this reagent accounts for the apparent increase in Table I. This is further confirmed by the higher absorption of the peak in the 440 mμ region. For example, based on the 440 peak 1.49 μmoles of "histidine" were recovered whereas based on the 570 mμ peak only 1.09 residues were found. The ninhydrin constant of ϕ'-cysteine (Figure 1) at 570 mμ is approximately 16 in a system in which lysine = 25 and half-cystine = 11.

The reaction of amino acids with ϕ' Br was examined under a variety of conditions. Results of a number of

ELUTION OF φ'-CYSTEINE ON 20-CM ANALYZER COLUMN
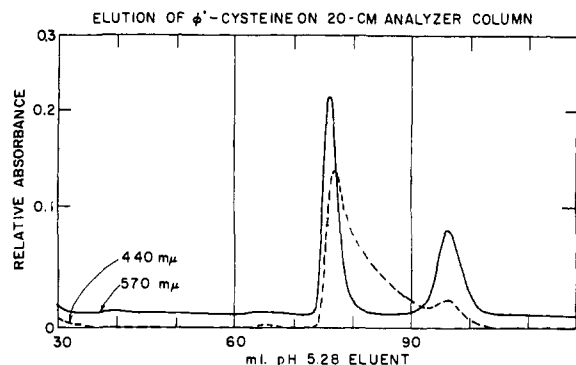
Figure 1. Elution of φ'-cysteine derivative on the 20-cm. column with pH 5.28 citrate buffer eluent. Solid line = absorbance at 570 mμ after reaction with ninhydrin. Symmetrical peaks at 76 ml. and 97 ml. are φ'-cysteine and ammonia, respectively. Absorbance at 440 mμ appearing in the column effluent between these peaks is due to 2-hydroxy-5-nitrobenzyl alcohol present in this sample, which is eluted in a broad peak at this position.

Figure 2. Spectra of φ' Br-treated ribonuclease and mercaptoethanol-reduced ribonuclease. (Curves are displaced with respect to vertical axis for display on common horizontal axis.) Conditions: ribonuclease 8 × 10⁻⁵ M, treated with 3 × 10⁻² M φ' Br at pH 5.0, in 8 M urea, 5% acetone.

experiments are summarized in Table II. Reaction of φ' Br with L-tryptophan was essentially complete when mixtures of amino acids were allowed to react with the reagent in a mole ratio of φ' Br to amino

Table II. Reaction of Amino Acids with 2-Hydroxy-5-nitrobenzyl Bromide (φ' Br) under Various Conditions[a]

Expt. no.	Amino acid, M × 10³	φ' Br, M × 10²	pH	—Apparent recoveries (μmoles)—		
				Amino acid	Control	φ' Br-treated
1	1.17	3.1	3.3	Try	1.00	0.00
				Cys	0.97	0.67
				His	1.00	(1.22)[b]
				Tyr	1.00	0.99
2	0.94	3.1	7.0	Try	1.00	0.005
				Cys	0.91	0.81
				His	1.00	(1.04)[b]
				Tyr	0.98	0.99
3[c]	1.1	3.1	11.5	Try	0.91	0.02
				Tyr	0.98	0.28
4	1.1	1.1	3.5	Try	1.00	0.00
				Cys	0.79	0.62
				His	1.00	(1.08)[b]
				Met	1.01	0.99
				Tyr	0.98	0.99
5	1.1	1.1	11.3	Try	1.00	0.10
				Cys	0.79	0.07
				His	1.00	(1.41)[b]
				Met	1.01	0.97
				Tyr	0.98	0.57
6	0.1	0.1	3.2	Try	0.98	0.00
				Cys	0.98	0.89
7	1.17	3.1	3.2	Try	1.01	0.00
				Cys	0.98	0.79
8[d]	1.17	3.1	3.2	Try	0.96	0.08
				Cys	0.95	0.81

[a] Conditions: see Table I. Amino acids other than those listed were unchanged in control and φ' Br-treated samples. [b] Values high because of presence of modified cysteine in histidine region as explained in text. [c] No cysteine added. [d] In 8 M urea.

acid of 10:1, over a range of reagent concentrations of 1.0 × 10⁻³ M to 3.1 × 10⁻² M. This was true in acidic, neutral, or basic solutions, and in the presence or absence of 8 M urea. In the neutral or acidic solutions the only other amino acid altered was cysteine
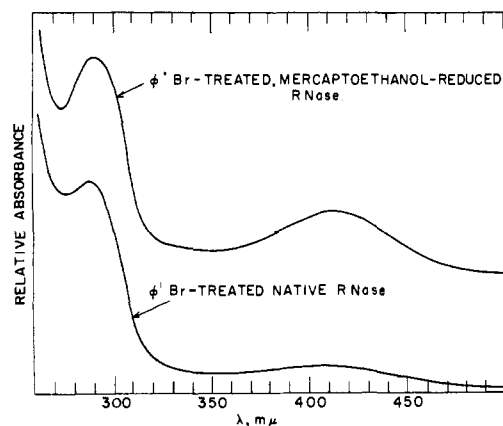
whose reactivity with φ' Br appeared to be no more than 20% that of tryptophan.

In alkaline solutions of an environment in which the hydroxyl group of tyrosine would be ionized, reaction with this amino acid was observed. Moreover, the reactivity of φ' Br with cysteine, which should also be ionized and hence more nucleophilic at this pH, was enhanced so that the reactivity of the cysteine and tyrosine residues appeared to be nearly equal to that of tryptophan.

These experiments with the free amino acids confirmed the previously reported conclusions as determined by experiments with chymotrypsin in neutral and acidic media. In view of this good agreement, it is probably correct to deduce that the reactivity in alkaline solutions determined from the free amino acids will apply to proteins as well.

Reaction of φ' Br with Ribonuclease. When φ' Br was allowed to react with chymotrypsin which contains seven tryptophan residues and no cysteine residues it was demonstrated that only tryptophan reacted with the reagent.[3] To establish that the results with free amino acid residues were applicable in protein studies, it seemed desirable to perform tests on ribonuclease. Ribonuclease seemed particularly valuable for this purpose since it does not contain any free cysteine[10] but the cystine linkages can be reduced to produce sulfhydryl residues. Moreover, this protein does not contain any tryptophan. In preliminary experiments Karkhanis[11] had shown that the native ribonuclease did not react with φ' Br to any appreciable extent and that reduced ribonuclease containing free sulfhydryl groups did react.

These experiments were confirmed in detail by the following experiment. Ribonuclease A, a protein containing 8 half-cystines linked in disulfide bridges, was treated at pH 5 in 8 M urea with 0.03 M φ' Br. A second batch of enzyme was reduced with mercaptoethanol according to the method of Anfinsen and Haber.[12] The protein in each case was separated from

(10) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **235**, 633 (1960).
(11) Y. D. Karkhanis, unpublished experiments.
(12) C. B. Anfinsen and E. Haber, *J. Biol. Chem.*, **236**, 1361 (1961).

excess reagent by gel filtration through 2 × 20 cm. columns of Sephadex G-25 (fine beads). The results are shown in Figure 2. The degree of binding of the 2-hydroxy-5-nitrobenzyl group was calculated by comparing the absorbance at 410 mμ in strong base relative to the protein absorbance at 290 mμ. No significant amount of chromophoric group was bound covalently to the native protein whereas approximately 0.2 mole of the phenolic chromophore was bound per mole of the reduced enzyme. This value is well below the groups bound per mole of chymotrypsin when treated with the same concentration of reagent in 8 M urea. It is surprisingly somewhat low for a protein containing 8 cysteine residues on reduction. Probably most of the cysteine residues are refolded into interior portions of the molecule and are inaccessible although some reoxidation may have occurred.

Thus the results with proteins are consistent with the reactivities of the free amino acids. In acid solution tryptophan and cysteine residues react with 2-hydroxy-5-nitrobenzyl bromide and at high pH's both of these, plus tyrosine react. No other amino acids yield stable products either in the protein or as the free amino acid.

Determination of the Amount of 2-Hydroxy-5-nitrobenzyl Groupings per Mole of Protein. In protein modification studies it is desirable to have a quick and easy method for determining the amount of reagent which has formed a covalent link with the protein. The simplest method of determining the total number of groupings per mole of protein is by the spectral absorption in the case of a chromophoric group. In the previously reported experiments with chymotrypsin,[8] it was found that there was a direct quantitative relationship between the moles of ϕ' covalently bound per mole of protein based on spectral data and on the amino acid analyses.

When the amino acids were treated with ϕ' Br under the conditions given in Table I and then subjected to conditions of basic hydrolysis (3.7 M NaOH, 16–24 hr. at 110°) no regeneration of tryptophan could be detected. This confirms the results with whole protein, *i.e.*, chymotrypsin, and established that the ϕ'-treated tryptophan is stable to alkaline hydrolysis.

The tryptophan derivative is firmly bound to the sulfonated resin at pH 5.28 and remains on the short column even after 24 hr. elution by the usual buffer arrangement for the amino acid analyzer. The derivative is easily removed upon washing with 0.2 N sodium hydroxide, the reagent used to regenerate the column under standard procedures.

To avoid laborious chromatography on the column, it would be desirable if tryptophan could be determined using the p-dimethylaminobenzaldehyde reagent of Spies and Chambers.[13] Experiments with this analytical procedure on a mixture of tryptophan and ϕ'-tryptophan gave results which were somewhat variable in color intensity. A contribution to the final color value which was not negligible came from the ϕ'-tryptophan. Thorough extraction of the aqueous solutions with diethyl ether prior to incubation of the sample with p-dimethylaminobenzaldehyde gave somewhat more consistent findings but failed to produce quantitative results. Since this colorimetric method

(13) J. R. Spies and D. C. Chambers, *Anal. Chem.*, 21, 1249 (1949).

could not be used for determining tryptophan without further study, the amino acid analyzer was used for tryptophan assays.

The cysteine derivative of ϕ' was found to appear under the histidine peak on the short column of the amino acid analyzer. As a result, it could not be used for quantitative determination of the cysteine derivative and other procedures were attempted. The performic acid oxidation method of Moore[14] appears applicable for ascertaining the degree of reaction of cysteine with ϕ' Br. When a solution of amino acids was treated with ϕ' Br and then subjected to performic acid oxidation under Moore's conditions, the analyses which are presented in Table III were

Table III. Estimation of ϕ' Br Reaction with Cysteine Using Performic Acid

	Oxygenated, not hydrolyzed		Performic acid 36 hr. HCl	
Amino acid	Control	ϕ' Br	Control	ϕ' Br
Aspartic acid	1.00	0.99	1.01	0.97
Alanine	1.01	1.01	1.02	1.03
Half-cystine	0.96	0.67	0.00	0.00
Cysteic acid	0.00	0.00	1.00	0.73
Phenylalanine	1.00	1.00	0.84	0.94
ϕ'-Cysteine, by difference	0.29		0.27	

obtained. The difference in cysteic acid between the control aliquot and that treated with ϕ' Br was equal to the cystine missing from the sample which had not been treated with performic acid. In the case of the performic acid oxidized and HCl treated sample, the ninhydrin-positive ϕ'-cysteine peak (which eluted 76 ml. after zero time from the 20-cm. column) was totally missing. Thus, although the ϕ' derivative of cysteine is not stable to conditions of performic acid oxidation and hydrolysis, such treatment does not result in the regeneration of cysteic acid. The cysteic acid values, therefore, can be used to determine the unreacted cysteine in the native protein.

To check this result in a protein the ϕ' derivative of reduced ribonuclease was examined. Table IV presents results comparing the amino acid analyses of ϕ' Br treated native ribonuclease and reduced ribonuclease. The results show that acid hydrolysis alone does not quantitate the cysteine reaction. However, the performic acid treatment showed a difference in cysteic acid recovery equal to the amount of 2-hydroxy-5-nitrobenzyl groups bound per mole of protein as detected by spectral absorption at 410 mμ.

An alternate method which was not actually tried, but which would appear to be theoretically sound, would involve the reaction of unchanged cysteine with iodoacetic acid. The iodoacetate should react with the unreacted sulfhydryl groups and not with the ϕ' derivatives. Acid hydrolysis under standard conditions should then lead to quantitation of the carboxymethyl cysteine values.

Reactivity of ϕ' Br with Tryptophan, Methionine, and Water. As a reagent, 2-hydroxy-5-nitrobenzyl bromide is unusual in several respects. Its specificity

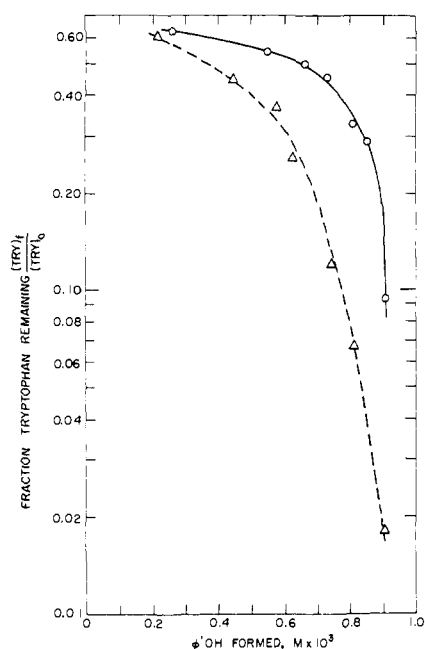(14) S. Moore, *J. Biol. Chem.*, 238, 235 (1963).

Figure 3. Reactivity of tryptophan with ϕ' Br in absence and presence of methionine. Conditions: see Table V. The results of Table V are plotted in the form of eq. 5. Continuous line: tryptophan at various concentrations treated with 0.001 M ϕ' Br; broken line: tryptophan at various concentrations treated with 0.001 M ϕ' Br in the presence of 0.001 M L-methionine. (In this type of plot, the points on the right-hand portion of the abscissa are the results of treating lower concentrations of tryptophan, while points on the left-hand portion represent higher initial concentrations of tryptophan.)

in modifying tryptophan is far greater than that of its analogs benzyl bromide and 2-methoxy-5-nitrobenzyl bromide,[15] yet it appears to be far more reactive than these analogs. Attempts to assess the rate of hydrolysis by following the release of HBr with 1 N NaOH in the autoburet of a Radiometer pH-stat assembly were unsuccessful. Delivery of the sodium hydroxide at top speed failed to be rapid enough to follow the rate of proton release when ϕ' Br was added to water at pH 5, even in the absence of added nucleophiles. From the limit of manipulations it could be concluded that the half-life of the reagent in water was less than 30 sec.

Although it was not possible to measure the hydrolysis rate of ϕ' Br directly, a further indication of the short half-life was obtained. In one experiment the ϕ' Br was added to water and mixed 30 sec. before tryptophan was added (see control sample in Table V). No reaction with tryptophan ensued under these circumstances, whereas complete reaction of tryptophan was shown to occur when tryptophan was present in the aqueous solution at the time of addition of ϕ' Br. Thus, the reaction of ϕ' Br with water must have been complete in less than 30 sec.

A reagent which reacts without any energy of activation should in theory react without specificity, i.e., it would react in proportion to the statistical distribution of the species in solution. Such behavior clearly cannot be the case for the 2-hydroxy-5-nitrobenzyl bromide and therefore a finite activation energy for reaction must exist even though the reaction appears

(15) H. R. Horton, H. Kelly, and D. E. Koshland, Jr., J. Biol. Chem., in press.

Table IV. Estimation of ϕ' Br Reaction with Cysteinyl Residues in Protein Using Performic Acid[a]

| | Recovery (μmoles/μmole RNAase) | | | |
| | 24 hr. HCl | | Performic acid | |
Amino acid	Native	Reduced	Native	Reduced
Lysine	10.1	10.2		
Histidine	3.9	3.9		
Arginine	4.1	4.1		
Cysteic acid	0.4	0.3	7.75	7.54
Aspartic acid	15.4	15.8	16.0	16.0
Meth. sulfone	0.0	0.0	4.0	3.9
Threonine	10.3	10.4	10.3	10.3
Serine	14.8	15.1	14.8	14.7
Glutamic acid	12.1	12.1	12.4	12.6
Proline	4.0	3.9	4.1	4.0
Glycine	3.0	3.0	3.3	3.3
Alanine	12.1	12.1	12.4	12.5
Half-cystine	6.7	7.5	0.0	0.0
Valine	8.8	8.6	8.9	9.9
Methionine	3.8	4.0	0.0	0.0
Isoleucine	2.3	2.4	2.5	2.6
Leucine	2.0	2.0	2.1	2.2
Tyrosine	5.8	5.6	0.0	0.0
Phenylalanine	3.0	3.0	2.8	2.9

ϕ'-Cysteine, by difference of cysteic acid = 0.21
ϕ' Bound to protein, by spectral measurement = 0.22

[a] Conditions: Native or reduced RNAase (8×10^{-5} M) + ϕ' Br (3×10^{-2} M), pH 5, 8 M urea, 5% acetone.

Table V. Effect of L-Methionine on Reaction of L-Tryptophan with ϕ' Br in Aqueous Solutions[a]

Initial concn., $M \times 10^3$		Fraction Try reacted	Fraction ϕ' Br reacted with Try
L-Try	L-Met		
0.10	0	0.906	0.091
0.10	1.0	0.982	0.098
0.20	0	0.714	0.143
0.20	1.0	0.933	0.187
0.286	0	0.681	0.195
0.286	1.0	0.880	0.251
0.50	0	0.547	0.274
0.50	1.0	0.744	0.372
0.667	0	0.499	0.333
0.667	1.0	0.635	0.423
1.00	0	0.454	0.454
1.00	1.0	0.551	0.551
2.00	0	0.370	0.740
2.00	1.0	0.393	0.786
2.00	2.0	0.413	0.826
2.00	4.0	0.421	0.842
5.0	0	0.199	0.99
10.0	0	0.10	1.00

[a] Conditions: 1.0×10^{-3} M ϕ' Br, 5% acetone, pH 3.

to be extremely rapid. An approximation of the relative reactivities of tryptophan and water is possible based on the observation that at a tryptophan concentration of 5×10^{-3} M essentially all of the tryptophan reacts with reagent. Using 55 M for the concentration of water, this means that the ϕ' Br reagent is 10,000 times more reactive with tryptophan than with water on a mole to mole basis.

A further anomalous feature of the 2-hydroxy-5-nitrobenzyl bromide is its failure to react with methionine, since both of its less reactive analogs, 2-methoxy-5-nitrobenzyl bromide and benzyl bromide, formed sulfonium derivatives with methionine. Since the 2-hydroxy compound was generally more reactive, it appeared that the failure to observe sulfonium forma-

tion was caused by decomposition of the sulfonium salt rather than failure to form this product in the first place.

To check this hypothesis, mixtures of methionine and tryptophan were allowed to react with ϕ' Br. The results are presented in Table V. The samples were extracted with ether to remove all 2-hydroxy-5-nitro-benzyl alcohol prior to adjustment of pH and addition of the sample to the analyzer. This treatment was found necessary since the evaporation and analysis procedure led to some reaction between the 2-hydroxy-5-nitrobenzyl alcohol (in the presence of HBr) and tryptophan. The results in Table V show that the presence of methionine at a concentration equal to that of the 2-hydroxy-5-nitrobenzyl reagent results in a larger proportion of reaction with tryptophan. It is clear, therefore, that methionine increases the reaction with tryptophan and the only reasonable explanation for this phenomenon is that the sulfonium salt of the ϕ' must be formed as an intermediate during the decomposition of ϕ' Br. This conclusion was reinforced by the synthesis of benzyl dimethyl sulfonium bromide[16] which reacted rapidly with tryptophan and water.

Discussion

Specificity of 2-Hydroxy-5-nitrobenzyl Bromide. Of the many features of the 2-hydroxy-5-nitrobenzyl bromide reagent, perhaps the most important is its specificity. Under acidic conditions the reagent reacts extremely rapidly with tryptophan and somewhat less rapidly with cysteine. Thus in proteins lacking free cysteine the reagent is specific for tryptophan from low pH up to pH values at which enzymes are usually active. Photooxidation and N-bromosuccinimide treatment continue to be valuable tools of the protein chemist since various reagents and overlapping specificities are needed in protein studies, but this new reagent of more restricted specificity should help to clarify and enhance studies with the oxidation reagents. Even in proteins containing cysteine, the ϕ' Br can be used to modify tryptophan residues selectively by protecting the cysteine residues reversibly, e.g., with bis-β-carboxyethyl disulfide as Stracher[17] has done for myosin.

The specificity can be explained by a combination of properties. Since indole rings are known to be attacked by alkylating agents and benzyl bromide reacts with tryptophan,[11] the 2-hydroxy-activated benzyl bromide would be expected to react readily with this amino acid. The lack of apparent reaction with methionine is explained (see above) by the decomposition of the sulfonium salt which is formed as an intermediate.

Failure to react with α-amino groups in proteins and with imidazole and other side chains is probably related to a competitive reaction between these nucleophiles, water, and OH⁻ ion. The reagent is extremely reactive with water. Therefore, any side chain in the protein must react unusually rapidly in order to compete successfully with the much greater concentration of water molecules. Moreover, as the pH is increased, making amino groups less protonated, the

concentration of hydroxide ion, an excellent nucleophile, increases. Apparently at the high pH only tyrosine and the mercaptide ion can compete with the water and OH⁻ ions.

Reactivity of 2-Hydroxy-5-nitrobenzyl Bromide. Usually high reactivity and selectivity are inversely related, i.e., a more reactive reagent has less selectivity. Although it is anomalous that 2-hydroxy-5-nitrobenzyl bromide contains both of these properties, the high reactivity, like the specificity, is explainable by reasonable chemistry. The compound 2-methoxy-5-nitrobenzyl bromide, which has the sluggish reactivity of benzyl bromide, contains the 5-nitro group.[15] Thus, it is clear that the nitro group is not responsible for the high reactivity. The hydroxyl group therefore appears to be responsible, and an explanation which has a solid basis in organic chemistry is shown in eq. 1. The quinoid resonance stabilizes the carbonium
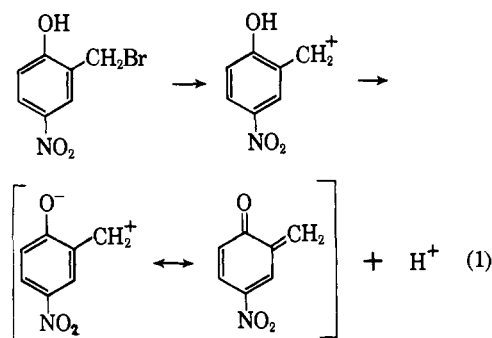
ion ultimately leading to rapid reaction. Filar and Winstein[18] have recently studied the properties of a number of *para* isomers capable of forming quinone methide structures. The quinone methide derivative of Filar and Winstein also showed a half-life of seconds in water similar to the half-life of this compound.[18] It is not established that a quinone methide intermediate occurs in these reactions (in fact, the effect of the leaving group on the reaction argues against exclusive formation of such an intermediate), but the type of resonance that stabilizes the quinone methide structure undoubtedly plays a role in the high reactivity of this reagent.

Mechanism of the Reaction with Tryptophan. To examine the special reactivity of this reagent, the kinetics of its decomposition were studied. A simple mechanism in which the ϕ' Br reacts with water and tryptophan in bimolecular events is shown in eq. 2.
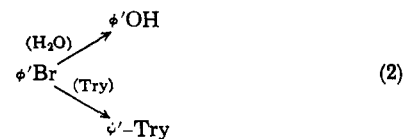
The rate expressions are given in eq. 3 and 4, from which

$$\frac{d(\phi' \text{ OH})}{dt} = k_w(H_2O)(\phi' \text{ Br}) \quad (3)$$

$$\frac{d(\phi'\text{-Try})}{dt} = k_{Try}(\text{Try})(\phi' \text{ Br}) \quad (4)$$

the integrated expression of eq. 5 can be derived. The subscripts 0 and f refer to initial and final concen-

(16) W. N. White, unpublished data.
(17) A. Stracher, *J. Biol. Chem.*, **239**, 1118 (1964).
(18) L. J. Filar and S. Winstein, *Tetrahedron Letters*, No. **25**, 9 (1960).

$$\ln \frac{(\text{Try})_f}{(\text{Try})_0} = -\frac{k_{\text{Try}}}{k_{\text{w}}(\text{H}_2\text{O})}(\phi' \text{ OH})_f \qquad (5)$$

trations, respectively. Since water concentration is constant during the reaction, this mechanism should give a straight line when the fraction of tryptophan remaining is plotted against the amount of 2-hydroxy-5-nitrobenzyl alcohol formed. As seen in Figure 2 the results do not correspond to a straight line and the departure from linearity indicates a higher reactivity at high trytophan concentrations than is expected on the basis of eq. 5.

A simple calculation for a mechanism involving two molecules of tryptophan as shown in eq. 6 leads to the relationship shown in eq. 7. Plotting the data in the

$$\frac{d(\phi' \text{ Try})}{dt} = k'_{\text{Try}}(\text{Try})^2(\phi' \text{ Br}) \qquad (6)$$

$$\frac{1}{(\text{Try})_f} - \frac{1}{(\text{Try})_0} = \frac{k'_{\text{Try}}}{k_{\text{w}}(\text{H}_2\text{O})}(\phi' \text{ OH})_f \qquad (7)$$

form of this equation leads to an even greater departure from linearity.

In view of the great reactivity of the reagent and its unusual selectivity and kinetics, it would appear that some type of complex between the ϕ' Br and tryptophan occurs prior to reaction. The rapid formation of a complex could explain the presence of both high reactivity and great selectivity in the same molecule. Such complexes have their analogies in charge-transfer complexes and the hydrophobic bonding between oily residues. Moreover, such a complex involving an aromatic ring might help to explain the high reactivity with tyrosine as well as with tryptophan.

In regard to the kinetics, it is perhaps worth mentioning that the leaving group must have an influence on the specificity of the reagent. This conclusion derives from the methionine enhancement of the reaction with tryptophan relative to water. If the 2-hydroxy-5-nitrobenzyl sulfonium intermediate partitioned between water and tryptophan in the same ratio as the 2-hydroxy-5-nitrobenzyl bromide, no enhanced reactivity with tryptophan would have been observed. The same would be true if the quinone methide were the exclusive reactive intermediate in both cases. The increased rate, therefore, not only established that a ϕ'-methionine intermediate exists, but that its tryptophan–water reactivity ratio is greater than that of the ϕ' Br. Presumably other leaving groups might be found which improve the selectivity of the reagent even further.

Application of the Reagent. The fact that ϕ' Br has a different specificity from the other tryptophan reagents, photooxidation and N-bromosuccinimide, means that analysis of results obtained after treatment by these various reagents may allow a mosaic which may lead to the explanation of the role of each residue.

Moreover, a reagent of high specificity is always a powerful tool in protein modification studies. Under appropriate circumstances, *e.g.*, in tryptophan-free proteins or when the roles of tryptophan have been ascertained, the reagent may also be useful for cysteine and tyrosine modification.

The high reactivity of the 2-hydroxy-5-nitrobenzyl bromide over a wide range of pH makes it of particular value in protein modification studies. Since this reagent reacts rapidly over a wide pH range, the pitfall of many reagents which require long incubation times at unfavorable pH is avoided. Secondly, the high reactivity with water means that excess reagent is destroyed, thereby minimizing the possibility of undesirable side reactions during the separation process.

Another feature of this reagent is its chromophoric group which absorbs in a region of the spectrum in which proteins are transparent. Thus the number of ϕ' groups absorbed per mole of chymotrypsin can be determined simply without the necessity of laborious amino acid analyses. The preferred pH to read this spectrum is greater than 10 where the extinction coefficient at 410 mμ is approximately 18,000 M^{-1} cm.$^{-1}$. When small amounts of the yellow reagent are covalently bonded to the protein, the number of ϕ' groups per mole of protein can be determined from the 410 mμ (base):280 mμ (acid) ratios. Since a large part of the protein absorption is contributed by the tryptophan and since the ϕ' Br reacts with tryptophan, it has been found that the 280 mμ peak is not a reliable index of protein when large amounts of the ϕ' reagent have been allowed to react. In that case, some other simple assay or an amino acid analysis may be required to determine protein concentration.

The application of this reagent has revealed that the ϕ' OH compound is readily absorbed to protein surfaces. The absorbed colored compound will give false high values for the number of residues which have reacted unless care is taken to remove it. In many cases Sephadex chromatography has been sufficient to remove the absorbed material, but in other cases it has been necessary to dissolve the protein in urea solution before gel filtration or acid precipitation to ensure the absence of the absorbed ϕ' OH. It is, of course, always possible to perform amino acid analyses in order to determine or corroborate the number of residues covalently attached to the protein.

Finally, the *p*-nitrophenolic grouping confers an absorption spectrum which is sensitive to environment and, therefore, this reagent can be used as a "reporter group" for studies of specificity and for conformational changes.[19]

(19) M. E. Burr and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.*, **52**, 1017 (1964)