Photogenerated Reagents for Biological Receptor-Site Labeling

JEREMY R. KNOWLES

The Dyson Perrins Laboratory, University of Oxford, Oxford, England

Received May 19, 1971

A very large number of important biological processes involve the interaction of a small organic molecule (the "ligand") with a biological macromolecule (the "receptor"). Enzymes catalyze reactions of their substrates and cofactors and the rates of these reactions may be changed by effectors; antibodies bind their haptens; hormones interact with their receptors; and macromolecular carriers transport ligands across biomembranes. In all these cases, we want to discover how the ligand and receptor interact at the molecular level.

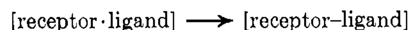
Spectroscopic methods such as fluorescence, esr, and nmr can provide useful information, but the size of many receptor molecules demands an often unattainable sensitivity of the spectroscopic technique if the ligand·receptor complex is to be observed directly. Moreover, most of the interesting interactions are reversible, and the necessity of having relatively high concentrations of ligand (to saturate the receptor) reduces the utility of spectroscopic approaches. Finally, these methods can rarely identify the actual constituents of a ligand binding site.

If, however, one presents the receptor with an analog of its natural ligand into which is incorporated a chemically reactive group, then the receptor binding site may be specifically tagged. If one does not know what the receptor is, then chemical tagging (*e.g.*, of the acetylcholine receptor in whole nerve, or of a transport protein in a cell membrane) with a ligand-like radioactive reagent may allow its isolation. For a purified and characterized receptor, the labeling allows one to identify what parts of the receptor macromolecule constitute the binding site.

The chemical approach to receptor site labeling therefore aims to simulate the natural situation

$$\text{receptor} + \text{ligand} \rightleftharpoons [\text{receptor}\cdot\text{ligand}]$$

with a reagent that structurally mimics the natural ligand and preserves the specificity of the interaction. If the reagent is not to label an irrelevant receptor, then these specificity requirements must be satisfied. In contrast to the necessary specificity of the binding interaction, the subsequent intracomplex reaction that labels the receptor covalently



$$[\text{receptor}\cdot\text{ligand}] \longrightarrow [\text{receptor–ligand}]$$

should be *non*selective. Ideally the reagent should react with the receptor site whatever its chemical constitution. The design and use of such indiscriminate reagents, and their advantages over established reagents of lesser reactivity, are discussed in this Account.

In enzymology, the reagent has been called[1] an "active-site-directed irreversible inhibitor," and its design is based upon the following criteria. (1) The reagent must contain the structural elements required for binding at the active site. It may often be that groups at the active site have, by the nature of their environment, enhanced chemical reactivities; but even if they do not, the high local concentration of reagent at the binding site should ensure preferential reaction. (2) It should not react rapidly with water nor too vigorously with the protein amino acids. These restrictions of reagent reactivity are obvious: a reagent subject to rapid hydrolytic breakdown is not useful,[2] nor is one which reacts so indiscriminately with amino acid groups that the effect of local concentration differences is nullified. (3) If possible, the reagent should be designed in the knowledge of the enzyme's probable mechanism. If the pathway of the enzyme reaction logically requires a group of a particular kind, then we may enjoy the conceptual bonus of designing a reagent explicitly to label the postulated functionality.

As one of a very large number of examples of this approach, consider the enzyme triosephosphate isomerase. This enzyme is responsible for the rapid interconversion of the trioses dihydroxyacetone phosphate and D-glyceraldehyde phosphate (Scheme Ii). Kinetic evidence and isotope labeling experiments led Rose[3] to propose a chemically reasonable first step for this reaction, in which an enzyme base abstracts a proton from the substrate, leading to an enediolate ion (Scheme Iii). With this in mind, we made the compound bromohydroxyacetone phosphate, which satisfies all the four criteria for active-site-directed inhibitors outlined above. The bromo compound stoichiometrically inactivates the enzyme very rapidly indeed (Scheme Iiii) and results in the attachment of the label to a unique glutamic acid residue in the protein.[4] This allows the tentative identification of "enzyme-B$^-$" (Scheme I) as a carboxylate group.

Jeremy Knowles graduated in chemistry from Balliol College, Oxford, in 1959, and worked for his D.Phil. with R. O. C. Norman on the mechanism of aromatic substitution. During a postdoctoral at Caltech in 1961, he succumbed to the lure of mechanistic enzymology, and returned to Oxford University to work on hydrolytic enzymes, particularly chymotrypsin and pepsin. More recently, he has studied the detailed mechanism of triosephosphate isomerase, and is currently investigating a number of glycolytic enzymes. He has been visiting professor in both Biophysics and Chemistry Departments at Yale, and is now University Lecturer in organic chemistry at the University of Oxford, and Fellow of Wadham College.

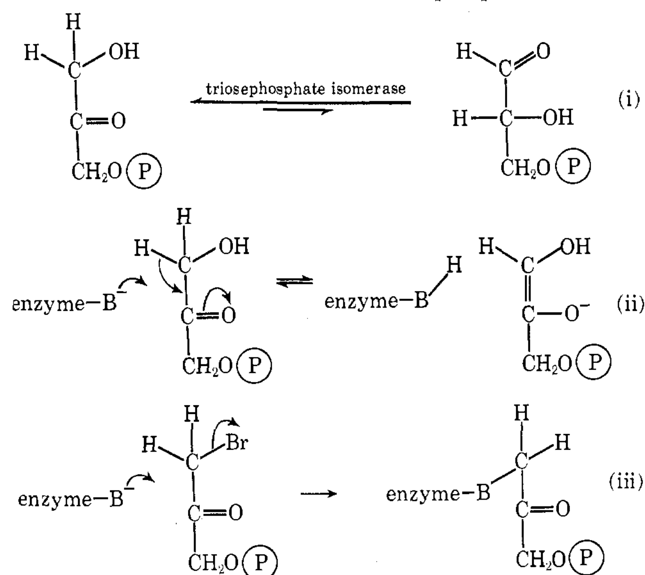
(1) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967; E. Shaw, *Physiol. Rev.*, **50**, 244 (1970).

(2) Though it may be in some cases; see *e.g.*, S. M. Parsons, L. Jao, F. W. Dahlquist, C. L. Borders, T. Groff, J. Raes, and M. A. Raftery, *Biochemistry*, **8**, 700 (1969).

(3) I. A. Rose, *Brookhaven Symp. Biol.*, **15**, 293 (1962).

(4) A. F. W. Coulson, J. R. Knowles, J. D. Priddle, and R. E. Offord, *Nature (London)*, **227**, 180 (1970); F. C. Hartman, *Biochemistry*, **10**, 146 (1971).

Scheme I
Active-Site-Directed Inhibition of Triosephosphate Isomerase



Labeling experiments of this kind tend, by the nature of the reagents used, to find *nucleophiles* at the active sites of enzymes.[5] This limitation has not been severe in enzymology, since *electrophilic* sites in proteins are normally cationic acids (and one cannot label protons) or metal ions (the existence and essentiality of which are often clear-cut), and the only other centers of interest in heterolytic chemistry are nucleophiles. But only about half of the 20 amino acids that may constitute an active site contain nucleophilic centers. If the technique of active site labeling is to be useful more generally for the study of biological receptors, for example, for receptor detection in membranes, in organelles, or in whole cells, reagents must be found that are capable of reacting even with "hydrocarbon" amino acids and with lipids.

Moreover, many biological receptor molecules do not *do* anything in chemical terms, but merely bind their ligand, and there is no *a priori* reason to expect reactive nucleophilic centers at the ligand binding sites. It may even be dangerous to use reagents selective for nucleophiles, since the affinity labeling technique relies on local concentration differences and misleading labeling patterns may arise in which the nearest reasonably accessible nucleophile is tagged.

Furthermore, any approach that is based upon a reagent used in aqueous solution is limited by the fact that water is more reactive than many of the functional groups in amino acid side chains. So it is hopeless to limit the chemical range of possible reagents to those having half-lives in neutral aqueous solution of longer than a few minutes. A reagent capable of attacking even carbon–hydrogen bonds is required. Clearly such a species cannot be added externally, and must be *generated from a stable precursor in situ.* What reactive species, then, can be generated, and what can be the generation process?

The only common chemical species capable of carbon–hydrogen bond insertion are carbenes and nitrenes. These can be produced thermolytically or photolytically. Thermal generation is ruled out by the small temperature range over which biological systems retain their structural integrity. Photolytic generation is, by contrast, ideal since by appropriate reagent design the photolysis can be carried out at wavelengths long enough to avoid most photooxidative or other radiation-induced damage to the system.

Carbene Generation

Carbenes can be generated by the photolysis of compounds such as diazoalkanes, diazirines, and α-keto diazo compounds, which produce the carbene on loss of nitrogen.[6] Carbene itself, produced from diazomethane, is strikingly reactive, and even at −75° shows little or no selectivity toward the different carbon–hydrogen bonds of *n*-pentane.[7] This behavior, however, is at least partly due to the excess vibrational energy that CH_2 possesses when generated from CH_2N_2. From ketene photolysis, carbene is more selective, and discriminates between secondary and primary carbon–hydrogen bonds 1.7-fold.[8] Carbomethoxycarbene shows a selectivity of 2.3, and dicarboethoxycarbene (from diazomalonic ester) is less reactive still, with a selectivity of 8.4.[9] Nevertheless, carbenes undoubtedly satisfy the need for an undiscriminating reagent.

For present purposes, three additional criteria must be met: the precursor must be chemically inert; the photolysis should be done at wavelengths clear of receptor absorption; and on photolysis, the immediate product should not rearrange to something less reactive. For a carbene, there is no reagent precursor that fulfills all of these conditions. α-Diazo ketones, esters, etc., are the most chemically stable and they are readily photolyzed on irradiation above 350 nm. Unfortunately, the α-ketocarbene so produced readily undergoes the intramolecular Wolff rearrangement to a ketene (in methanol solution, between 20 and 60% of the carbene from diazo esters rearranges to ketene[10]). Indeed, irradiative decomposition of α-diazo ketones is often used to effect the Wolff rearrangement in "difficult" cases.[6] The splendidly indiscriminate nature of the carbene is then largely lost, ketenes being subject principally to attack by nucleophiles.

The Use of Carbenes

The germinal experiment in the field of photogenerated reagents was done in 1962 by Westheimer and his group,[11] who reported the preparation and photolysis of monodiazoacetyl-α-chymotrypsin. *p*-Nitrophenyl

(5) B. L. Vallee and J. F. Riordan, *Annu. Rev. Biochem.*, **38**, 733 (1969); L. A. Cohen, *ibid.*, **37**, 695 (1968).

(6) T. L. Gilchrist and C. W. Rees, "Carbenes, Nitrenes and Arynes," Nelson, London, 1969; W. Kirmse, "Carbene Chemistry," Academic Press, New York, N. Y., 1964.

(7) W. v. E. Doering, R. G. Buttery, R. G. Laughlin, and N. Chaudhuri, *J. Amer. Chem. Soc.*, **78**, 3224 (1956).

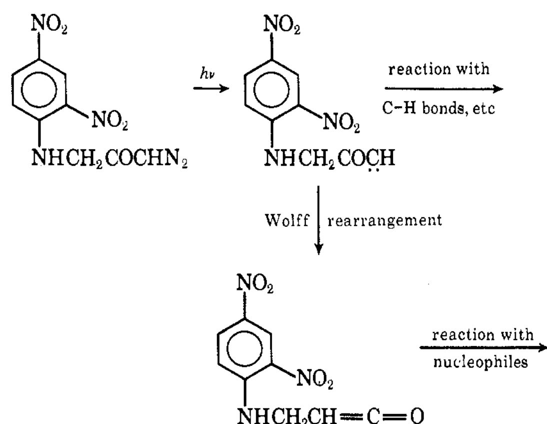
(8) H. M. Frey and G. B. Kistiakowsky, *ibid.*, **79**, 6373 (1957).

(9) W. v. E. Doering and L. H. Knox, *ibid.*, **83**, 1989 (1961).

(10) H. Chaimovich, R. J. Vaughan, and F. H. Westheimer, *ibid.*, **90**, 4088 (1968).

(11) A. Singh, E. R. Thornton, and F. H. Westheimer, *J. Biol. Chem.*, **237**, PC3006 (1962); J. Shafer, P. Baronowsky, R. Laursen, F. Finn, and F. H. Westheimer, *ibid.*, **241**, 421 (1966).

Scheme II
Carbene Labeling of Anti-dinitrophenyl Antibody



[^{14}C]diazoacetate was allowed to inactivate the enzyme by acylation of its active site serine-195. The isolated monodiazoacetyl-enzyme was then photolyzed (λ >350 nm), and after hydrolysis the labeled amino acids were identified. Three factors militated against the complete success of this experiment. First, the photogenerated carbene was produced on the surface of the protein, and the extraordinarily high reactivity of the carbene led to a large amount (50–60%) of product resulting from reaction with water. Secondly, the intrinsic chemical reactivity of the diazoacetyl group led to products arising from chemical (as distinct from photochemical) reactions with neighboring groups. Thirdly, some of the carbene underwent a Wolff-type rearrangement resulting in an alkoxyketene that hydrolyzed to *O*-carboxymethylserine. In summary, about 40% of the diazoacetyl group remained attached to protein, though only about 3% was from the hoped-for intramolecular carbene insertion reactions.

Subsequently Vaughan and Westheimer[12] produced the carbene from the [^{14}C]diazomalonyl half-ester of trypsin by irradiation at 254 nm and obtained [^{14}C]-glutamic acid after hydrolysis of the irradiated acyl-enzyme. This must have come from carbene insertion into the methyl side chain of an alanine residue and represents the first successful labeling of a hydrocarbon amino acid in a protein. The yield of the insertion product was very small (1–3%), but the feasibility of the photogeneration approach is put on firm ground by this work.

More recently, Converse and Richards[13] have obtained promising results using a photogenerated carbene to map the binding site of a specific antibody. In immunochemistry the need for unselective reagents is much more acute than in enzymology since, as has been pointed out, there is no reason to expect chemically functional (and certainly not chemically reactive) groups at the binding site. The approach of Converse and Richards is illustrated in Scheme II. Using an antibody against the 2,4-dinitrophenyl group, they added (*e.g.*) *N*-dinitrophenylglycine diazo ketone and irradiated the [antibody·reagent] complex between 300 and 400 nm. About 50% of the antibody binding sites were covalently labeled by this procedure. Although about half of this arises from reaction of ketene rearrangement product (see Scheme II), useful mapping information should emerge from this study, particularly from the products arising from reaction of the carbene itself. Further work is in progress[14] with the dinitrophenyl-binding mouse myeloma proteins MOPC 460 and 315, which have the advantage over normal antibodies in that they are molecularly homogeneous.

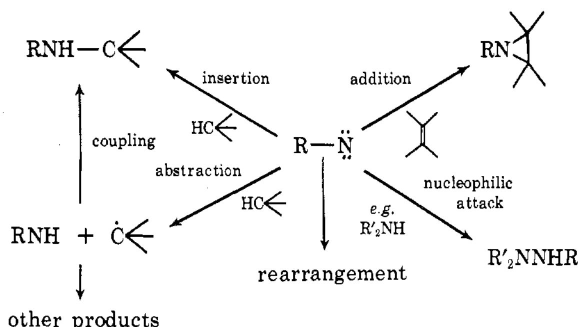
An analogous approach using a diazoacetyl precursor has been used to attack the acetylcholine receptor sites at the endplate of mouse diaphragm. Waser, *et al.*, showed that irradiation of isolated neuromuscular junctions in the presence of 10^{-5} *M* diazoacetylcholine bromide[15] caused irreversible depolarization of the post-synaptic membrane.[16] Isolated acetylcholinesterase was only 20% inhibited by a 50-fold higher concentration of reagent, indicating that the cholinesterase active site and the cholinergic receptor site are independent loci. Experiments of this kind may allow the isolation and characterization of the acceptor molecule, avoiding both the tedium and the uncertainty of following isolation by binding assay only.

Nitrene Generation

Nitrenes may be produced photolytically by reactions analogous to those forming carbenes, from alkyl and aryl azides and isocyanates and acyl azides. In general, nitrenes are more selective than carbenes. Whereas carbomethoxycarbene (produced by irradiation of methyl diazoacetate) reacts with tertiary alkyl carbon–hydrogen bonds only about three times faster than with primary carbon–hydrogen bonds,[9] the corresponding difference for carbethoxynitrene (from azidoformic ester) is 27-fold.[17] In the latter case the selectivity of the reagent is independent both of the solvent and of the method of generation. Similarly, the rates of attack on secondary carbon–hydrogen bonds and primary carbon–hydrogen bonds in *n*-pentane by phenylcarbene from phenyldiazomethane differ by 6-fold,[18] yet for phenylnitrene the difference is more than a 100-fold.[19] These differences in reactivity are reflected, as expected, in the relatively long lifetime of arylnitrenes, which are in the region of 10^{-4} sec for simple nitrenes in a "soft" polystyrene matrix.[20] Arylnitrene half-lives vary with substituent and increase

(12) R. J. Vaughan and F. H. Westheimer, *J. Amer. Chem. Soc.*, **91**, 217 (1969).
(13) C. A. Converse and F. F. Richards, *Biochemistry*, **8**, 4431 (1969).
(14) F. F. Richards, private communication.
(15) J. Frank and R. Schwyzer, *Experientia*, **26**, 1207 (1970).
(16) P. G. Waser, A. Hofmann, and W. Hopff, *ibid.*, **26**, 1342 (1970).
(17) W. Lwowski and T. J. Marcich, *J. Amer. Chem. Soc.*, **86**, 3164 (1964).
(18) C. D. Gutsche, G. L. Bachman, and R. S. Coffey, *Tetrahedron*, **18**, 617 (1962).
(19) J. H. Hall, J. W. Hill, and H. Tsai, *Tetrahedron Lett.*, 2211 (1965).
(20) A. Reiser, F. W. Willets, G. C. Terry, V. Williams, and R. Marley, *Trans. Faraday Soc.*, **64**, 3265 (1968).

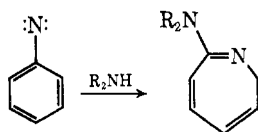
Scheme III
Possible Fates of Nitrenes



by nearly two orders of magnitude on going from *p*-acetyl (short-lived) to *p*-*N*-morpholino (long-lived).[21] A range of reagents of different reactivities can therefore in principle be synthesized.

The reactions open to a nitrene are abstraction (normally of hydrogen from carbon), cycloaddition, direct insertion (usually into carbon–hydrogen bonds), attack by nucleophiles, and rearrangement (see Scheme III).[6] Abstraction reactions followed by radical coupling will lead to insertion, and while direct intermolecular insertion reactions are rare, insertion products are common in intramolecular reactions. If a nitrene is generated *in situ* at a binding locus, the intramolecular situation is probably a more appropriate model. Direct insertion, abstraction–coupling, or addition reactions will then result in covalent attachment of label to the site.

Rearrangement reactions can, as with carbenes, reduce the effectiveness of the reagent. Acyl azides, were their use not ruled out on chemical reactivity grounds, rearrange smoothly on photolysis (*cf.* the Curtius and Schmidt rearrangements) to isocyanates, analogously to the Wolff rearrangement of α-dizao ketones to ketenes. Even in cases stereochemically favorable for intramolecular insertion by acylnitrene, some 70% of isocyanate results.[22] *Aryl*nitrenes are much less susceptible to rearrangement,[23,24] the major rearrangement path resulting in substituted azepines. This ring expansion may result in the attachment of label to neighboring nucleophiles,[23] as



Of the three criteria for usable precursors (chemical inertness, lack of rearrangement of reactive entity, suitable absorption maxima), the first two are satisfied for nitrene generation only by some alkyl and most aryl azides. Alkyl azides have absorption maxima around 290 nm, however, and it is not possible to effect photolysis (for some systems, at least[25]) without damage to the receptor. So we are left with aryl azides which are chemically stable at 37°, not drastically susceptible to photochemical rearrangement, and which can—if appropriately substituted—be photolyzed to the arylnitrene at wavelengths above 350 nm.

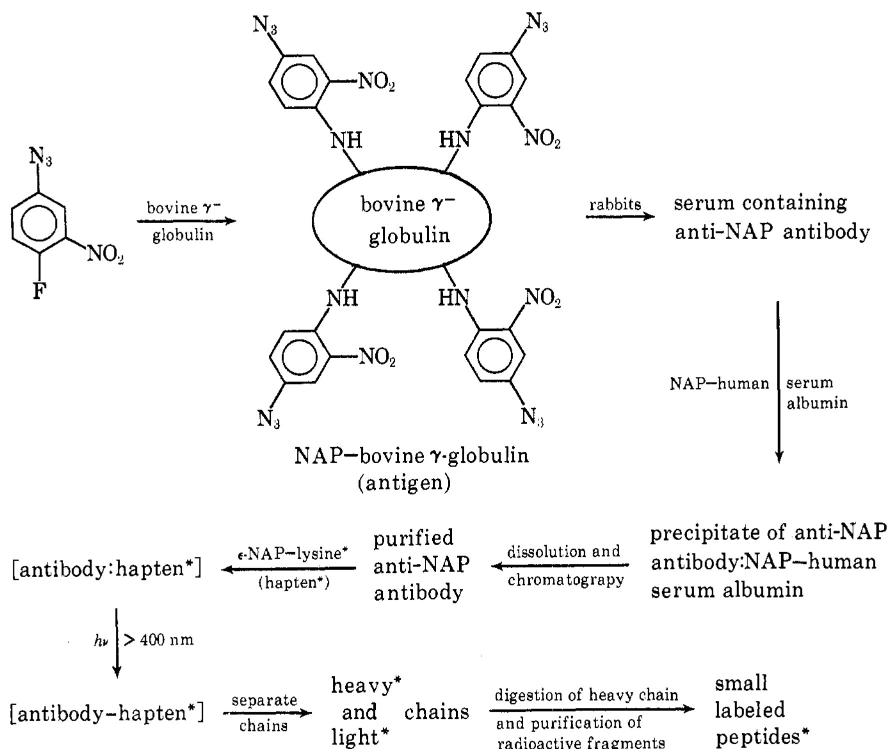
The Use of Nitrenes

An arylnitrene was first used as a labeling reagent for a specific antibody.[26] The use of an antibody as a system for investigation has two important advantages. First, [antibody·hapten] complex dissociation constants can be very small (10^{-7} *M* or less), which means that the protein can essentially be titrated with the reagent, and nonspecific reaction by reagent not in the binding site can be minimized. Westheimer and his colleagues overcame the problem of nonspecific reaction by prior covalent attachment of their carbene precursor,[11,12] but this approach is only appropriate to a few systems. Secondly, the specific antibody protein is selected by the immunized animal to fit the reagent, and one is not constrained by the structural limitations of, for instance, an enzyme's natural substrate. These considerations allow the exploitation of the animal to synthesize a protein appropriate for the reagent. Previous studies on antibodies had relied on cross-reaction (*i.e.*, the structural similarity between reagent and antigenic determinant), in which the reactive group was not itself likely to be part of the specifier characteristics of the antigen.[27]

In Scheme IV the main steps in the preparation and labeling of rabbit anti-NAP antibody are shown (NAP, 2-nitro-4-azidophenyl). The nitro group was included in the specifier not only to facilitate preparation of antigen (compare the use of dinitrofluorobenzene) but for two other reasons. First, the substitution shifts the λ_{max} of the hapten into the visible, away from the ultraviolet absorption of the protein itself. Secondly, a nitro group will decrease the half-life (*i.e.*, increase the reactivity) of the nitrene.[21] After irradiation of the [antibody·hapten] complex, some 65% of antibody sites were blocked by the specific covalent attachment of hapten.[28] Most of the label was found in the heavy chains, and after peptide digestion and purification of some of the smaller peptide fragments, two radioactive peptides, X-Ala-Arg and Phe-Cys-Y-Arg, were isolated. X and Y are modified amino acids of presently unknown structure.

(21) A. Reiser and L. Leyshon, *J. Amer. Chem. Soc.*, **92,** 7487 (1970).
(22) J. W. ApSimon and O. E. Edwards, *Can. J. Chem.*, **40,** 896 (1962).
(23) W. v. E. Doering and R. A. Odum, *Tetrahedron*, **22,** 81 (1966).
(24) C. Wentrup and W. D. Crow, *ibid.*, **26,** 3965 (1970).
(25) J. R. Knowles, unpublished work; I. A. Tarkchanova, S. S. Schanin, and A. Y. Kulberg, *Biochim. Biophys. Acta*, **175,** 464 (1969).
(26) G. W. J. Fleet, R. R. Porter, and J. R. Knowles, *Nature (London)*, **224,** 511 (1969).
(27) See, *e.g.*, S. J. Singer and R. F. Doolittle, *Science*, **153,** 13 (1966).
(28) There are various reasons why *all* the binding sites are not blocked. Possibly some of the covalent links formed are chemically labile, or were formed to water in the site, and in any case, 100% occupancy was not expected since in order to maximize the signal-to-noise ratio, the radioactive hapten was not in excess. More recently, using antibody from single allotype rabbits, lower extents of covalent labeling have been observed. However, affinity chromatography nicely separates protein molecules having about 80% of the binding sites covalently labeled by hapten from antibody molecules that still bind hapten noncovalently but which cannot be blocked by irradiation of the complex (E. M. Press, unpublished results).

Scheme IV
Preparation and Labeling of Anti-NAP Antibody[a]



[a] NAP, 2-nitro-4-azidophenyl; asterisks indicate tritium-labeled material.

Information of this kind is not very helpful by itself, but a study of the sequence of the heavy chain of pooled normal IgG by Porter and his group[29] revealed in positions 91–94 the sequence: –Phe-Cys-Ala-Arg–. It therefore appears probable that X is a modified cysteine residue and that Y is a modified alanine residue. However, more significant than the apparent success of this indiscriminate reagent in identifying a part of the antibody combining site, residues 91–94 are adjacent to the major "hypervariable" region of the heavy chain. That is, we have strong support for the premise that one of the hypervariable regions in the IgG molecule is at least partly responsible at the molecular level for antibody specificity.

It is worth pointing out that, had labeling occurred within the hypervariable region, we might have failed to find it. Presumably there is a large number of sequences arising from residues 95–105 which could preclude the isolation of adequate quantities of material of unique sequence from this region. It may be that, only because the reagent lifetime was long enough to allow labeling of a number of residues, it was possible to obtain analyzable amounts of material from the relatively constant region close by. We are currently investigating this point by using an analogous reagent to label one binding locus of a homogeneous protein of known tertiary structure: the tosyl hole of α-chymotrypsin.[30] This will answer the question whether one, or several, residues are tagged. It is already apparent that essentially all the label is in the C chain of the enzyme, which constitutes this specificity pocket in α-chymotrypsin.[31]

Recently, Kiefer, *et al.*,[32] have reported the use of an arylnitrene in the inactivation of acetylcholinesterase of erythrocyte membranes and in the labeling of the acetylcholine receptor of intact frog sartorius muscle. These workers showed that the precursor azides bind specifically to the sites, and that inactivation of the receptors was effected only by photolysis of the bound precursor. The utility of the method was apparent from the ineffectiveness, at correspondingly low concentrations (10^{-6} *M*), of analogous conventional diazonium salt reagents. Moreover, the use in these experiments of intact muscle shows that the method is viable even in the presence of overlying tissue. Aside from reagent design, the specificity for a particular site may be increased (this being necessary when, as with acetylcholine, at least two different receptors are present in the same area at the synapse) through the use of selective protecting agents. The use of microbeams of light to localize inactivation morphologically has also been suggested.[32]

The NAP reagent is also yielding very promising results in the labeling of protein and lipid components of cell membranes, being used initially as a probe of membrane topology by Richards and Staros.[33]

(29) R. G. Fruchter, S. A. Jackson, L. E. Mole, and R. R. Porter, *Biochem. J.*, **116,** 249 (1973); L. T. Mole, S. A. Jackson, R. R. Porter, and J. M. Wilkinson, *ibid.*, **124,** 301 (1971).
(30) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, **46,** 337 (1969).
(31) R. J. Foster, unpublished work.
(32) H. Kiefer, J. Lindstrom, E. S. Lennox, and S. J. Singer, *Proc. Nat. Acad. Sci. U. S.*, **67,** 1688 (1970).
(33) F. M. Richards and J. Staros, private communication.

Photooxidative Approaches

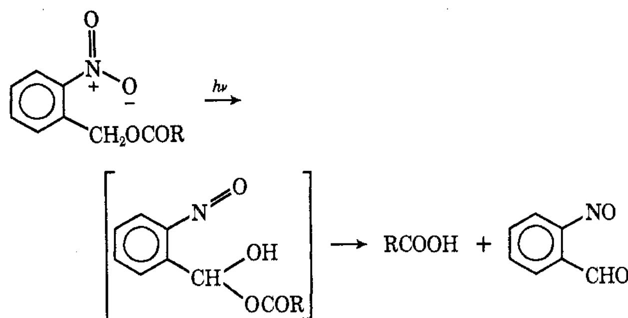
Developing out of the use of dye-sensitized photooxidation as a means of selective modification of proteins,[34] the application of chromophoric substrate-like materials as active-site photosensitizers has recently been reported.[35,36] Oxidation is normally restricted to rather few amino acids (Cys, Trp, Tyr, Met, His), and of course the modification is not useful in labeling terms unless the protein is small enough to allow an assessment of *absent* residues. An example of the possible use of a photogenerated free radical (at the active site of α-chymotrypsin, from the hydroperoxy-[Ser_{195}]-enzyme) as a chemical probe has also been reported.[37]

Photodeprotection

For some purposes, it may be appropriate to liberate a chemically functional group (as distinct from a highly reactive species) at a particular site in a biological system. For instance, one may want to generate a fluorescent molecule inside, or on the "wrong" side, of a biomembrane, and photodeprotection reactions are potentially useful in this area.

Deprotection reactions fall into two classes. Examples of the first group all involve internal redox reactions and intramolecular transfer of oxygen. The second group of reactions relies on the increased likelihood of heterolytic breakdown or of solvolytic cleavage of excited states. Deprotection of three functional groups have been described.

For carboxylic acids, the internal redox pathway was first exemplified by Barltrop and his group,[38] who showed that *o*-nitrobenzyl esters readily liberate acid on photolysis:



(34) L. Weil and T. S. Seibles, *Arch. Biochem. Biophys.*, **54**, 368 (1955); E. W. Westhead, *Biochemistry* **4**, 2139 (1965); J. S. Bellin and C. A. Yankus, *Arch. Biochem. Biophys.*, **123**, 18 (1968).
(35) M. Rippa and S. Pontremoli, *ibid.*, **133**, 112 (1969); E. Scoffone, G. Galiazzo, and G. Jori, *Biochem. Biophys. Res. Commun.*, **38**, 16 (1970); G. Gennari, G. Jori, G. Galiazzo, and E. Scoffone, *J. Amer. Chem. Soc.*, **92**, 4140 (1970).
(36) F. Sawada, *J. Biochem. (Tokyo)*, **65**, 767 (1969).
(37) M. J. Gibian, D. L. Elliott, and W. R. Hardy, *J. Amer. Chem. Soc.*, **91**, 7528 (1969).

The use of 2,2′-dinitrobenzhydryl esters has also been reported.[39] Barton has proposed[40] the use of 2,4-dinitrobenzenesulfenyl esters and suggested that these compounds undergo heterolysis on irradiation, to yield the sulfenyl cation and the carboxylate anion.

For protected amino groups, cleavage of excited states seems to occur readily, and irradiation of benzyloxycarbonyl,[41] 3,5-dimethoxybenzyloxycarbonyl,[42] or 3-nitrophenyloxycarbonyl[43] protected amines has been reported. Additionally, internal redox cleavage using substituted *o*-nitrobenzyloxycarbonyl groups also produces good yields of free amine on irradiation above 320 nm.[39]

Finally, nitrophenyl phosphates have been cleaved photolytically, in the order *m*-nitro > *o*-nitro > *p*-nitro.[44]

Conclusion

While the technique of photogeneration of reagents designed to label biological receptor sites is still young, it appears that the ability externally to generate reactive species at specific binding sites is a useful addition to the armory of reagents. Enough information is available to allow the design of reagents of proper specificity that will respond to irradiation of reasonably low energy and that have half-lives appropriate to the nature of the labeling problem.[45]

The author is grateful to Drs. Rodney Porter and George Fleet, in collaboration with whom the use of nitrenes was developed, to many colleagues for stimulating discussion and criticism, and to the Medical Research Council and Science Research Council for financial support.

(38) J. A. Barltrop, P. J. Plant, and P. Schofield, *Chem. Commun.*, 882 (1966).
(39) A. Patchornik, B. Amit, and R. B. Woodward, *J. Amer. Chem. Soc.*, **92**, 6333 (1970).
(40) D. H. R. Barton, Y. I. Chow, A. Cox, and G. W. Kirby, *J. Chem. Soc.*, 3571 (1965).
(41) J. A. Barltrop and P. Schofield, *Tetrahedron Lett.*, 697 (1962); *J. Chem. Soc.*, 4758 (1965).
(42) J. W. Chamberlin, *J. Org. Chem.*, **31**, 1658 (1966).
(43) Th. Wieland, C. Lamperstorfer, and C. Birr, *Makromol. Chem.*, **92**, 279 (1966).
(44) E. Havinga, R. O. De Jongh, and W. Dorst, *Recl. Trav. Chim. Pays-Bas*, **75**, 378 (1956).
(45) NOTE ADDED IN PROOF. Recent work on the products from the photolysis of ethyldiazomalonyl-chymotrypsin and trypsin [Hexter and Westheimer, *J. Biol. Chem.*, **246**, 3934 (1971)] and of diazoacetyl-chymotrypsin [Hexter and Westheimer, *ibid.*, **246**, 3928 (1971)] has elucidated the nature of products of both intra- and intermolecular reaction of the carbene. Westheimer's group has also reported the synthesis of a coenzyme analog, 3-diazoacetoxymethyl-NAD$^+$, that labels yeast alcohol dehydrogenase [Browne, Hixson, and Westheimer, *ibid.*, **246**, 4477 (1971)]. Promising photosensitive analogs of cyclic-AMP have also been made and used with phosphofructokinase [Brunswick and Cooperman, *Proc. Nat. Acad. Sci. USA*, **68**, 1801 (1971)].