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High yield photoreagents for protein crosslinking and affinity labeling

(nitrophenyl ethers/phototransfer substitution reactions/maleimides/fetal hemoglobin/oxygen affinity)

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ABSTRACT **4-Nitrophenyl ethers are proposed as new high-yield photoreagents for protein crosslinking and affinity labeling. These are totally unreactive in the dark under biological conditions, but react quantitatively with amines at pH 8 upon irradiation with 366-nm light. The reaction of monoalkoxy-*p*-nitrobenzenes with an amine yields the corresponding free alcohol and substituted nitrophenylamine. In essence, the nitrophenyl group is transferred from the alcohol to the amine. Bifunctional affinity reagents of this type could be especially useful for placing the *p*-nitrophenyl chromophore adjacent to a binding site without blocking it. The corresponding 2-methoxy-4-nitrophenyl ethers react with amines by displacement of the methoxyl group. Thus, bifunctional reagents of this class could be photocrosslinkers. A maleimide-containing 2-methoxy-4-nitrophenyl ether was attached to human fetal hemoglobin at γ-cysteine F9 stoichiometrically. Subsequent ultraviolet irradiation yielded a γ-γ crosslinked hemoglobin in 80% yield. The oxygenation properties of the derivative indicate that it is locked in a high affinity conformation and that all cooperativity is lost.**

Crosslinking and affinity labeling of biological macromolecules have been used with increasing frequency for structural and functional studies. The first reagents employed in these techniques were derived from common acylating and alkylating reagents (1, 2). The fate of such reagents in solutions of biological macromolecules is not easily controlled. In some cases, the reagent may suffer extensive hydrolysis before reaching the macromolecule. If it reaches a target, it is likely to react at the first opportunity, freezing the system and denying experimental access to later stages.

One solution to this problem is to place potential reactive groups in an inert form which can be activated at the proper time. In biological systems, the most convenient way to trigger a reaction *in situ* is near-ultraviolet photoirradiation (3, 4). Aliphatic, resonance-stabilized diazo compounds (5) and aromatic azides (6) were the first photoreactive groups to be tried. However, the carbenes and nitrenes generated from such materials have serious drawbacks. Their extremely high reactivity, which allows them to bind covalently to almost any macromolecular component, also allows them to react efficiently with water or buffer components. For this reason, the observed yields of covalent products are often very low (3), although some exceptions are known (7–9). Recently, aromatic ketones have shown promise as potential photoactivatable reagents, producing higher yields of desired products (4, 10). In many instances the usefulness of crosslinking reagents is also limited by problems encountered during the identification of crosslinked products. Many analytical techniques require the regeneration of the individual macromolecules. Cleavable crosslinking reagents that allow such regeneration include disulfides (11), glycols (12), and bisimidoesters (13, 14).

From these and other considerations, an ideal affinity or crosslinking reagent would be unsymmetrical with one of the reactive groups present as a photoactivatable precursor. Photolysis would lead to high yield reaction with macromolecular targets but to no reactions with buffer components. The length of the reagent would be variable (by using a series of homologues) to allow adjustment of the reach of the reactive ends. Provision should be made for the eventual cleavage of crosslinked pairs under mild conditions. Here we shall describe a set of reagents that meet most of the above criteria. They are based on the nucleophilic aromatic photosubstitution reactions of nitrophenyl ethers (15–19).

MATERIALS AND METHODS

Photolyses. Typical photoreactions were carried out at 4° using two long-wavelength ultraviolet lamps (366 nm) equipped with Sylvania F8TS tubes. The lamps were positioned face-to-face, 2 cm on either side of a rack containing parafilm-stoppered glass tubes (13 × 100 mm). Unless otherwise specified, the buffer used for all reactions was either 50 or 100 mM sodium phosphate, pH 8.0. Quantitative model reactions (15) were studied in 1-cm quartz UV cells with a Perkin–Elmer MPF-2A spectrofluorimeter at 315 or 366 nm.

Photoreagents. Detailed synthetic procedures and product characterization will be presented elsewhere.

N-Carbobenzyloxyglycine-4-nitrophenoxy ethyl ester was prepared by condensing 2-(4-nitrophenoxy)ethanol with *N*-carbobenzyloxyglycine using *N*,*N*′-dicyclohexylcarbodiimide. Cleavage with 30% HBr in acetic acid at 0° yielded 2-(4-nitrophenoxy)ethyl glycine ester. Reaction of this with *N*-hydroxysuccinimide esters of carbobenzyloxyglycine and -diglycine yielded, after deblocking, the respective 2-(4-nitrophenoxy)ethyl di- and triglycine esters.

The preparation of NGM_n and NPM_n is illustrated schematically in the text. The sodium salt of 4-nitrophenol and the potassium salt of 4-nitroguaicol were used in the original condensation with ω-bromonitriles. The reaction of the resulting ω-(4-nitroaryloxy) cyanoalkanes with hydroxymethylmaleimide was performed by crushing together 1 mmol of each, adding 1 ml of cold concentrated H_2SO_4, Vortex mixing, and allowing the mixture to stand at 4°, preferably overnight. Then the sample was added dropwise to ice and the desired *N*(maleimidomethyl)-ω(4-nitroaryloxy)carboxamido-alkane precipitated. It can be recrystallized from acetone.

The products were pure by thin layer chromatography (silica gel; benzene/ethanol, 9:1). They gave satisfactory NMR and microanalysis (20). Typical melting points were NGM_3, 173–174° and NGM_6, 139–141°.

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Abbreviations: NPM_n, *N*(maleimidomethyl)-ω(4-nitrophenoxy)carboxamidoalkane containing n methylene groups; NGM_n, *N*(maleimidomethyl)-ω(2-methoxy, 4-nitrophenoxy)carboxamidoalkane containing n methylene groups.

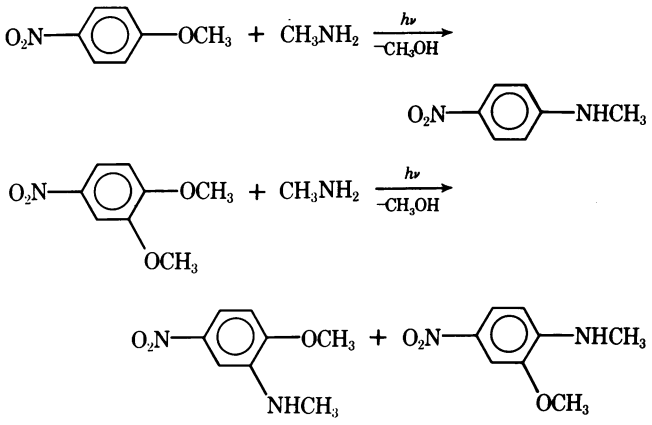
Reaction with Proteins. NGM_n in dry dimethylformamide solution was added to the protein in an appropriate nonreactive mercaptan-free buffer at pH 8. After a 30 min incubation at 4° in the dark, the protein solution was freed from excess reagent either by dialysis (three times with 1000-fold volumes for 2 hr each) or by passage through a Sephadex G75 column (Pharmacia Fine Chemicals). These operations were done under subdued light in a darkroom.

Hemoglobin. Human fetal hemoglobin was prepared from samples of cord blood (21). Electrophoresis was performed in 10% acrylamide gels (22) in the presence of sodium dodecyl sulfate. Gels were stained for 2 hr at 50° with a 0.1% solution of Coomassie brilliant blue R250 in 40% 2-propanol and 10% acetic acid. They were destained overnight in 10% acetic acid at 50° in the presence of a strand of undyed wool yarn. This affords a completely clear background. Amino acid analysis was performed on a JEOL 6AH analyzer (23). Samples were hydrolyzed for 24, 48, and 72 hr, and amino acid concentrations were extrapolated to limiting values. The analytic procedure gave poor quantitation of free cysteine, but provided excellent resolution of the product of the reaction of cysteine with maleimides obtained after acid hydrolysis, *S*-cysteinylsuccinic acid. Oxygen equilibrium measurements were performed tonometrically (24) and analyzed spectrophotometrically (25).

RESULTS

Studies on model systems

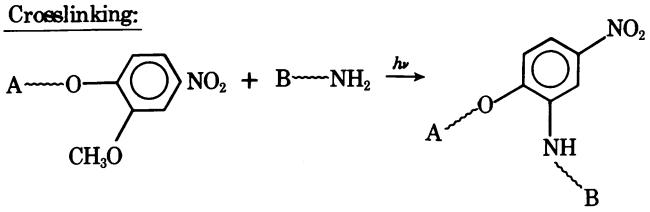
Several properties of nitrophenyl ethers make them attractive as potential biological photoreagents. Both 4-nitroanisole and 4-nitroveratrole are indefinitely stable in the dark at room temperature in aqueous solution even in the presence of common nucleophiles. They are stable, even upon irradiation, in the absence of nucleophiles (18). However, irradiation in the presence of primary amines leads to the substitution reactions:



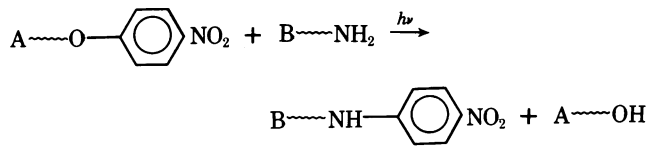
These reactions occur via an extremely short-lived triplet state (15). The observed lifetimes, 10^{-7} to 10^{-9} sec, are very short compared with the 10^{-3}-sec lifetimes of typical aromatic nitrenes (26). The short lifetime will ensure that the photoactivated species does not wander away from its proper target before reacting (27).

A second major advantage of the photoreaction of nitrophenyl ethers with amines is yield. Although the quantum yield of the reaction is not extremely high and depends upon the nucleophile concentration, the chemical yield and the conversion to product are both high. Nonproductive deactivation of the excited state simply regenerates the starting material. This is in sharp contrast to the destructive deactivation of nitrenes and carbenes. A scheme for exploiting the photochemistry of nitrophenyl ethers involves first attaching the ether to a macromolecule or ligand, denoted A in the schemes below. This derivative of A is added to a biological system and the photochemical reaction is triggered.

Crosslinking:



Label transfer:



The most likely reaction products at mildly alkaline pH will involve lysine side chains of a protein component, denoted as B. Lysine side chains are usually accessible from the solvent and show great freedom of motion (28). They tolerate even bulky substituents relatively well without apparent damage to biological activity (29–31).

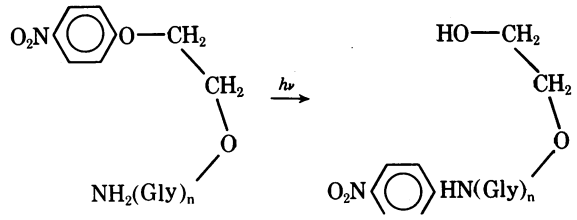
The first type of reaction crosslinks components A and B via a nitroanisidine-like bridge. The second type results in the transfer of the nitrophenyl moiety from A to B. Taken together, the two reactions can circumvent the need for cleavable adduct of A and B. The transfer mode would produce a derivative of B modified only by the addition of nitrophenol. Thus, it could allow for a variant of affinity labeling in which only part of the affinity probe is transferred to the target. The original ligand, A, would be free to leave. In principle, the target site could still accept additional ligands. The nitrophenyl moiety placed near the target site could be used for spectroscopic studies of subsequent events at that site.

Model reactions were investigated to study the photochemical behavior of nitrophenyl ethers under the conditions required by biological systems. By using methylamine and 4-nitroanisole in buffered aqueous solutions, we confirmed that the reaction products did not depend on the irradiating wavelength (32). For practical reasons, most subsequent studies were performed with 366-nm light. The photoreaction is conveniently monitored by the appearance of the intense 400-nm absorption band of 4-nitroaniline derivatives. At pH 8, a reasonably comfortable value for most proteins, a significant reaction occurs with methylamine and glycine ethyl ester. This pH was used for further work. All twenty common amino acids react at the α-amino group with 4-nitroanisole under such conditions, as evidenced by the disappearance of the starting material. In the case of glycine, the product was identified as *N*(4-nitrophenyl)glycine by comparison with authentic material (33).

Side reactions are evidenced by sulfur-containing and aromatic amino acids. Thiols (cysteine, *N*-acetyl cysteine and also mercaptoethanol) react with 4-nitroanisole about 20 times faster than methylamine at pH 8. The reaction products are not the known 4-nitrophenyl thioethers, indicating that thiols do not behave like amines with respect to this reaction. NH_2-blocked derivatives of methionine react to give a complex mixture of products, but disulfides (thioctic acid or *N,N'*-diacetylcystine) are essentially stable. Tryptophan reacts to yield a mixture of products, one of which exhibits a yellow fluorescence when irradiated at 366 nm. Tyrosine decomposes partially during

irradiation, even in the absence of nitroanisole, presumably by air oxidation. All other common amino acids (except lysine) that are acylated on the α-amino group are unreactive under the same conditions. Acetate, chloride, bromide, iodide, and nitrite are also unreactive.

In affinity labeling reactions, the reactive probe is held in the neighborhood of its target. A model system was constructed that allowed the comparison of simple, similar inter- and intramolecular reactions. An intramolecular model reaction is



The effect of nucleophile concentration on the rate of product formation in this system is compared with the bimolecular reaction of 4-nitroanisole and glycine ethyl ester in Fig. 1. The concentration of the photochemical partner in the reactions, the first excited triplet of the nitrophenyl ethers, was kept essentially constant, because at even the lowest concentration almost all the incident light was absorbed by the chromophore (>99.7%) and the reaction was observed at low conversion to minimize absorption by the product. The concentration dependence of the intermolecular reaction is typical of a bimolecular process. The rate of product formation increases with increasing nucleophile concentration until a saturation point is reached beyond which the reaction rate is presumably diffusion controlled (15). Under similar conditions, however, the reaction of the nitrophenoxyethyl esters of oligoglycine is independent of concentration, and the rate of product formation is about an order of magnitude greater than the corresponding bimolecular rate. This is consistent with an increase

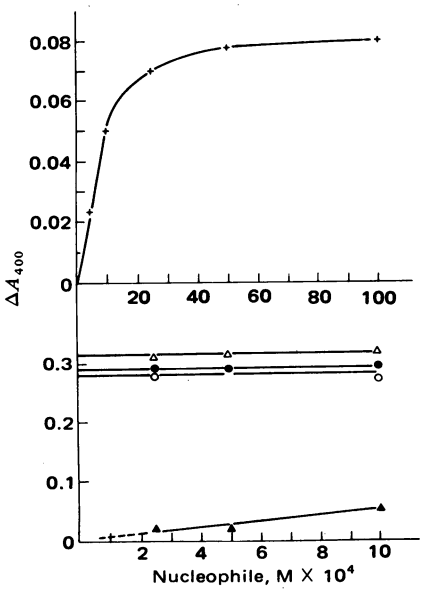


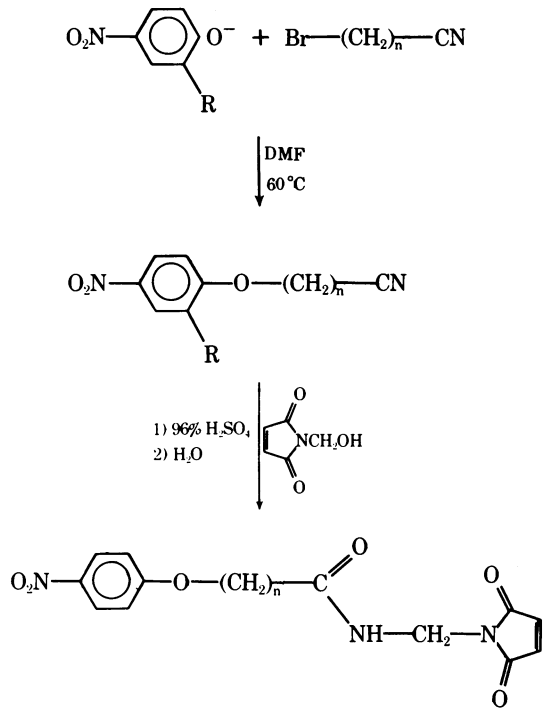
FIG. 1. Product formation as a function of nucleophile concentration. Irradiation was at 315 nm for 1 hr at pH 8. (*Upper*). The intermolecular reaction of glycine ethyl ester with 5×10^{-4} M 4-nitroanisole. (*Lower*). Comparison of the product formation by inter- and intramolecular reactions as a function of nucleophile concentration. ▲, Glycine ethyl ester plus 5×10^{-4} M 4-nitroanisole. Note 1:10 dilution compared with the data in *Upper*. ○, NH_2-Gly-O-$(CH_2)_2$-O-4-nitrophenyl; ●, NH_2-$(Gly)_2$-O-$(CH_2)_2$-O-4-nitrophenyl; △, NH_2-$(Gly)_3$-O-$(CH_2)_2$-O-4-nitrophenyl.

in the "effective" concentration of nucleophile experienced by the nitrophenyl ethers held in the neighborhood of the nucleophilic amino group, as has been observed in other intramolecular reactions between groups held in close proximity (34).

Mercaptoethanol (1 mM), which completely quenches the intermolecular reaction, still allows the intramolecular one to proceed at about 30% yield. Thus, under conditions of crosslinking or affinity labeling, one should expect significant reaction with amino groups unless a thiol is present in the immediate vicinity of the reactive moiety.

Application to a macromolecular system

To test this approach in a biological system, a family of reagents, NPM_n and NGM_n, was developed. The nonphotochemical reactive group is a maleimide, a well established thiol reagent. Most proteins contain few free sulfhydryl groups; therefore such a reagent will be relatively selective in its binding. The photochemical (nitrophenyl or nitroguaiacyl ether) reactive group is linked to the maleimide via a series of appropriately substituted aliphatic nitriles according to the scheme below.



NPM_n R = H

NGM_n R = OCH_3

Hemoglobin is a convenient system for testing these reagents. Human fetal hemoglobin consists of two α chains identical with the adult chains and two γ chains instead of the adult β chains. Recent studies have confirmed that the three-dimensional structures of crystalline hemoglobins A and F are virtually identical (35). The differences in sequence between the β and γ chains make fetal hemoglobin particularly useful for these studies: (36) the NH_2-terminal residue of the γ-chains is glycine, whereas that of the α and β chains is valine. The γ chains have only one sulfhydryl, cysteine F9(93)γ, which is stable under normal conditions and is reactive towards the usual sulfhydryl reagents. It is located near the interface between the γ subunits at a position expected to be sensitive to the conformation alterations that accompany deoxygenation (37).

Human fetal oxyhemoglobin reacted rapidly with NGM_3 and NGM_6 at pH 8 in phosphate buffer to yield a derivative prior

Table 1. Amino acid compositions of human fetal hemoglobin and its crosslinked and irradiated products

Amino acid	Theoretical chain compositions			Experimentally observed compositions*		
	α	γ	$(\alpha+\gamma)/2$	HbF	NGM_3-HbF	Dimer
Asp	12	13	12.5	12.4	13.0	12.8
Glu	5	12	8.5	8.9	9.2	11.1
Gly	7	13	10.0	10.8	10.6	12.4
Ala	21	11	16.0	15.6	15.7	12.4
Val	13	13	13.0	15.0	15.3	14.7
Ile	0	4	2.0	2.2	2.3	4.1
Leu	18	17	17.5	17.5†	17.5†	17.0†
Tyr	3	2	2.5	3.0	0.7	1.9
Lys	11	12	11.5	12.5	12.4	12.3
His	10	7	8.5	9.3	9.1	5.6
SucCys‡	0	1	0.5	0.0	0.3	0.8

* HbF, native human fetal hemoglobin; NGM_3HbF, fetal hemoglobin reacted with NGM_3 but not subjected to photolysis; dimer, 32,000 M_r band isolated by gel electrophoresis from photolyzed NGM_3HbF.

† The values were normalized with respect to leucine; the low values for tyrosine are unexplained.

‡ The theoretical yield of S-cysteinylsuccinic acid (SucCys) is 0.0 in the γ chains of native HbF and 1.0 in the γ chains of NGMHbF, assuming that only the "reactive" sulfhydryl group is alkylated by the maleimide ring. The sulfhydryl group of the α chain reacts with maleimides only when hemoglobin is denatured or dissociated into monomeric chains.

to irradiation in which the maleimide ring clearly reacted with the sulfhydryl side chains of cysteine, as shown by amino acid analysis (see Table 1) and as subsequently confirmed by use of a ^{14}C-labeled analog of NGM_n.

Photolysis of NGM_n-labeled hemoglobin purified from excess reagent by gel filtration of Sephadex G-75 yielded, in addition to monomeric chains, crosslinked products that were predominantly dimeric species when analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate (see Fig. 2). Some traces of trimers and tetramers were also observed, especially after prolonged irradiation. Molecular weight assignments of these species were confirmed by the use of hemoglobin chemically crosslinked with dimethysuberimidate as internal molecular weight electrophoresis standards. All the photocrosslinked products appear to result from intramolecular reactions because their yield and distribution was unaffected by a 10-fold variation in protein concentration.

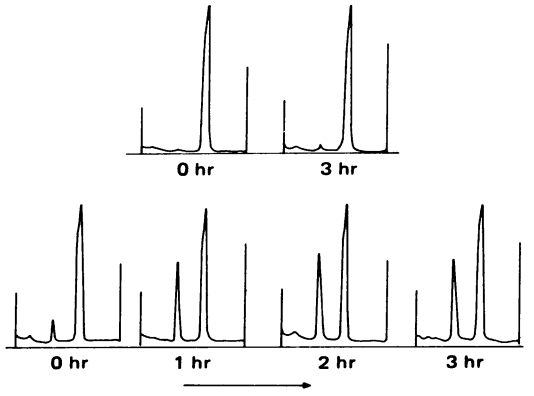


FIG. 2. Influence of the length of the irradiation time (366 nm at pH 8) on the crosslinking of NGM_3-labeled human fetal hemoglobin (HbF). Shown are scans of Coomassie blue-stained sodium dodecyl sulfate electrophoresis gels. (*Upper*). Irradiation of unlabeled HbF (control). (*Lower*). Irradiation of NGM_3–HbF. The HbF was labeled stoichiometrically.

We determined the yield of the various crosslinked species by scanning gels stained with Coomassie blue and normalizing the area of the resulting integrated peaks to 100%. A plot of product yield as a function of the ratio of reagent added per γ chain is shown in Fig. 3. The maximal yield of dimer was about 40%. Interestingly, the reaction appears to be saturated at NGM_6/γ chain = only 0.5:1. Thus, although two reagent molecules can bind per hemoglobin tetramer, only one crosslink is required to give the maximal yield of nondissociable dimer.

Dimers were purified by gel filtration in the presence of sodium dodecyl sulfate and subjected to amino acid analysis. The results shown in Table 1 indicate that the dimer consists solely of γ-chain pairs. The yields of glycine, alanine, glutamic acid, and isoleucine are especially diagnostic. Thus the maximal possible yield of dimer was only 50% on a chain basis. Since only one covalent bond between the chains was required to create a dimer, one molecule of reagent per hemoglobin tetramer sufficed. Of the two cysteine residues in each crosslinked dimer, 1.6 side chains reacted with the maleimide ring of the reagent, but we cannot determine from this analysis whether the dimers were actually crosslinked by one or two bridges. The γ-chain cysteines [F9(93)γ] accounted quantitatively for all the modified sulfhydryls found in the unphotolyzed NGM hemoglobin, indicating that the reaction was confined to these side chains, as expected from reaction with other maleimides. The maximal observed yield of 40% dimer on a chain basis represents 80% of the theoretical yield. This is quite a respectable value for a photochemical crosslinking. Additional studies are needed to determine the actual site hit by the photosubstitution.

The photocrosslinked hemoglobin tetramers are produced in high enough yield to enable preliminary functional studies. NGM_3-crosslinked human fetal hemoglobin exhibited a high affinity for oxygen and essentially no cooperativity (Table 2). This result suggests that crosslinking prevented the conformational changes that occurred during deoxygenation. On the basis of the known structure of hemoglobins, we expect that the crosslink spanned the cleft of the γ-γ interface. If it prevented the widening of this cleft, this would have locked the tetramer in the high affinity conformation, whether the hemes were liganded or not. Alternatively, significant perturbation of the conformation of the protein at the sites of nitrophenylation of the γ chains might eliminate cooperativity even if the crosslinks were long enough to span the γ-γ interface in the low affinity

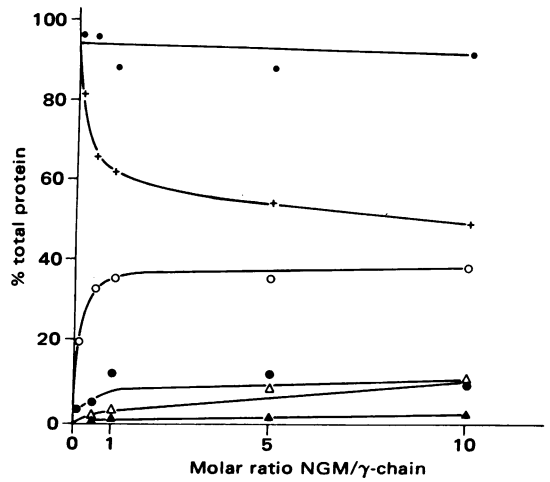


FIG. 3. Yield of the various oligomeric species obtained by crosslinking of human fetal hemoglobin at different ratios of NGM_3 to γ chains. Irradiation at 366 nm for 2.5 hr at pH 8. Irradiated samples are denoted by hν; control samples were not irradiated. •, Monomer (control); +, monomer (hν); ○, dimer (hν); ●, trimer (hν); △, dimer (control); ▲, tetramer (hν).

Table 2. Oxygenation equilibria for various hemoglobins*

	$\log P_{50}$	n
Hb A†	1.0	2.8
Hb F	0.75	2.8
NES Hb†	0.60	2.4
NPS Hb†	0.9	2.0
BME Hb	0.10	1.0
NES des Arg Hb†	−0.12	1.2
NGM_3HbF	−0.14	1.1
Free α or β†	−0.8 to −0.3	1.0

NES and NPS are hemoglobin reacted with NEM and nitrophenylmaleimide, respectively; BME Hb is hemoglobin crosslinked with bis maleimidomethyl ether; NES des Arg Hb is hemoglobin treated consecutively with carboxypeptidase B and NEM.

* Hill plot characteristics: n is the slope of the curve $\log [\overline{Y}/(1 - \overline{Y})]$ vs. $\log P_{O_2}$ at $\overline{Y} = 1/2$, P_{50} is the corresponding value of P_{O_2} in mm Hg. Experimental conditions are described in ref. 39.

† Published values are from Baldwin (37), Tyuma and Shimuzu (38), and Simon *et al.* (39).

deoxy conformation. Such extensive conformational perturbation has been observed in a derivative of hemoglobin A in which the NH_2-termini of the α chains are blocked with dinitrophenyl groups (40).

DISCUSSION

The efficient crosslinking of oxyhemoglobin with NGM justifies the choice of the nucleophilic aromatic photosubstitution reaction as the basis of protein crosslinking reagents. The usefulness of the NPM_n transfer reagents appears quite promising from model studies. Applications to macromolecular systems have thus far been severely hampered by the low specific activity of radioactive nitrophenol available. Tritiation by catalytic exchange could provide a solution to this problem.

The high reaction yields obtained, under mild conditions of pH and irradiation, promise a wide applicability of reagents built along similar lines, whether for crosslinking or affinity labeling. In particular, the chemical inertness of nitrophenyl ethers to all but the most drastic conditions can allow the synthesis of numerous reagents incorporating most of the known protein (and eventually nucleic acid or lipid) reagents. Imidoesters, organomercurials, and hydrazides have already been prepared (20). Many small molecules such as coenzymes, antibiotics, enzyme substrates, are amenable to chemical modification and can easily be conjugated to a nitrophenyl ether in order to construct affinity probes.

Such modifications as cleavable links using glycols, azo bonds, β-ketoesters or amides, and amidomethyl alkyl thioethers, should introduce an even greater flexibility in the use of such reagents. In addition, antibodies against the nitroaromatic haptens can be expected to considerably extend the range of possible uses of the reagents through the use of immunological techniques, affinity chromatography and electron microscopy.

We wish to thank Dr. Gilbert Stork for suggesting the series of photochemical reactions used in this work and Dr. Arnold Stern, Department of Pharmacology, New York University School of Medicine for samples of cord blood. Support for this research was provided by the U.S. Public Health Service in grants GM 14825 (to C.R.C.) and HL-13527 (to S.R.S.), the American Heart Association (76-688, to S.R.S.), and the New York State Health Research Council (HRC-40, to S.R.S.). S.R.S. is a National Institutes of Health Career Development Awardee (HL-00088).

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