BIFUNCTIONAL IMIDOESTERS AS CROSS-LINKING REAGENTS

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Received April 29, 1966

Bifunctional small molecule reagents have been important in the study of a number of problems in protein chemistry (cf. Wold, 1961 a, b; Zahn and Meienhofer, 1958; Singer, 1959). For most purposes it is desirable that the chemical modifications produced by the bifunctional reagent cause as little alteration as possible in the conformation of the protein molecule. The exhaustive amidination of ϵ-NH_3^+ groups with monofunctional imidoesters (Hunter and Ludwig, 1962) preserves the positive charges near these sites, and was shown to produce little or no significant changes in the conformational properties or biological activities of proteins (Wofsy and Singer, 1963). It was therefore anticipated that bifunctional imidoesters would be highly useful cross-linking agents, in that they would not affect the charge distribution of the proteins which they modified. Preliminary investigations along these lines are reported in this paper.

MATERIALS AND METHODS

Most of our studies were carried out with the reagent diethyl malonimidate dihydrochloride $C_2H_5O\text{-}C(=NH_2^+Cl^-)\text{-}CH_2\text{-}C(=NH_2^+Cl^-)\text{-}OC_2H_5$ (DEM[1]). This was prepared

[1] Abbreviations used: DEM, diethyl malonimidate dihydrochloride; BSA, bovine serum albumin; HGG, human γ-globulin; RGG, rabbit γ-globulin; anti-DNP, antibodies directed to the 2,4-dinitrophenyl hapten; DNPNS, 2-(2,4-dinitrophenylazo)-1-naphthol 3, 6-disulfonic acid, disodium salt.

from malononitrile and HCl in anhydrous ethanol-dioxane according to McElvain and Schroeder (1949), taking care to protect the system at all stages from moisture. The product melted at 134°-135° [literature values 119°-120°, and 138° (McElvain and Schroeder, 1949)] and its analysis was: calculated C, 36.52; H, 6.96; N, 12.17; Cl, 30.67; found C, 36.06; H, 6.82; N, 12.34; Cl, 30.35. The solid dihydrochloride was stored at -20°C. The stability of this reagent under a variety of aqueous solution conditions was estimated by an extraction and titration procedure closely analogous to that used by Hunter and Ludwig (1962) for monofunctional imidoester hydrochlorides, taking advantage of the fact that the free imidoester base is much more soluble in chloroform than in water.

Ethyl acetimidate hydrochloride was made as previously described (Wofsy and Singer, 1963).

The reaction of DEM with a protein was carried out as follows. To 0.24 gm solid DEM at 0°C was added with stirring 0.2 ml 5N NaOH, and 0.2 ml of the appropriate buffer; the pH was then raised to the desired value with 1 N NaOH. The protein to be modified, dialyzed against the appropriate buffer, was then added to make the final reaction volume 5 ml (0.2 M in DEM) and to contain the desired concentration of protein. If two proteins were to be cross-linked (BSA and HGG, or ferritin and HGG), their dialyzed stock solutions were added in rapid succession to the DEM solution to give a total volume of 5 ml. After the desired reaction time at 0°C, the mixture was generally dialyzed against phosphate buffer, pH 7.5, Γ/2 0.1.

DEM-modified anti-DNP antibodies were titrated with the dye hapten, DNPNS (Metzger, Wofsy, and Singer, 1963) in order to determine their residual specific binding activity.

The reaction of DEM with sheep red cells was carried out in a manner similar to that with the soluble proteins, using 0.5 ml of washed packed red cells in the pH 8.5 reaction mixture of 5 ml total volume. Reaction proceeded for 24 hours at 0°. Two control experiments were carried out. In

one, unmodified red cells alone were placed in the pH 8.5 buffer for 24 hours at 0°C. In the other, ethyl acetimidate hydrochloride and red cells were reacted under the same reaction conditions as were used with DEM. The three cell populations were then centrifuged and washed with phosphate-buffered saline, with results indicated in the next section.

The DEM-modified cells were tested for their capacity to undergo immune hemolysis in the presence of guinea pig complement and rabbit anti-sheep red cell antiserum, using a standard procedure (Wasserman and Levine, 1961).

The extent of amidination of individual proteins with DEM was measured by ninhydrin analyses of their remaining free amino groups, using the procedure of Schick and Singer (1961).

To ascertain whether the cross-linking of two proteins was produced by reaction with DEM, immunoelectrophoresis experiments were carried out. The protein mixture was electrophoresed at a total protein concentration of 10 mg/ml in the phosphate buffer. As a control, the two proteins were reacted separately under the same conditions and then mixed prior to immunoelectrophoresis.

Ultracentrifuge experiments were carried out in a Spinco Model E instrument at 59,780 r.p.m. near room temperature.

The BSA, horse spleen ferritin, RGG and rabbit antiserum to ferritin, were obtained from the Pentex Co., Kankakee, Illinois. The HGG was a Squibb product. The rabbit antiserum to HGG was kindly donated by Dr. R. W. Dutton. Rabbit anti-BSA sera and pure anti-DNP antibodies were prepared by standard procedures (Wofsy and Singer, 1963; Farah, Kern, and Eisen, 1960). Sheep red cells, and rabbit anti-sheep red cell sera were obtained from Hyland Laboratories, Los Angeles, and guinea pig complement was prepared by Dr. Stanley E. Mills.

RESULTS

1) Stability of the Reagent: Only rough estimates were made of the stability

of DEM to serve as a guide for the modification experiments. After 4 hours at 0°C at pH 7.5 or 8.5, the reagent was about 45% or 55% decomposed, respectively. After 21 hours at 0°C at pH 8.5, about 90% decomposition had occurred.

2) Reactions with Individual Proteins: Most of the experiments were carried out with BSA and γ-globulin separately under conditions that were found to produce cross-linking between them in their mixtures (see below). At pH 8.5 and 0°C, DEM blocked about 86% and 70% of the free amino groups of BSA (in 6% solution) and HGG (in 3% solution), respectively, in 4 hours. After 48 hours at pH 9.5 and 0°C, the extent of the reaction was raised to 94 and 86%, respectively. In two experiments with BSA carried out for only 30 minutes at pH 8.5 and 0°C, at 1.5 and 20% protein concentrations, the degree of blocking was 77 and 62%, respectively. Self-coupling of BSA or HGG was not extensive at concentrations between 1 and 6%, but could be greatly increased by increasing the protein concentration (fig. 1). After 1 hour reaction, a 20% BSA solution formed a solid gel which could not be dispersed on dilution. If this reaction was terminated earlier, soluble BSA aggregates were formed (fig. 1).

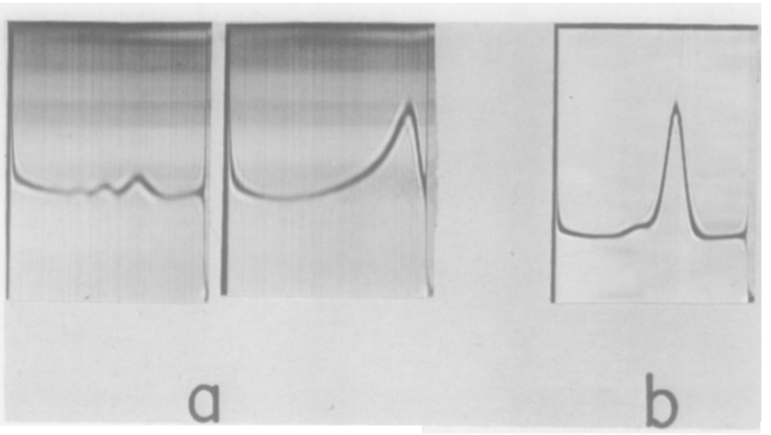


Figure 1. Ultracentrifuge patterns of DEM-treated BSA reacted at a) 20% and b) 1.5% protein concentration. Sedimentation is to the left. The patterns in a) were taken at 10 minutes (right) and 66 minutes (left) and b) at 74 minutes after reaching 59,780 r.p.m. Both samples examined at 1% protein concentration.

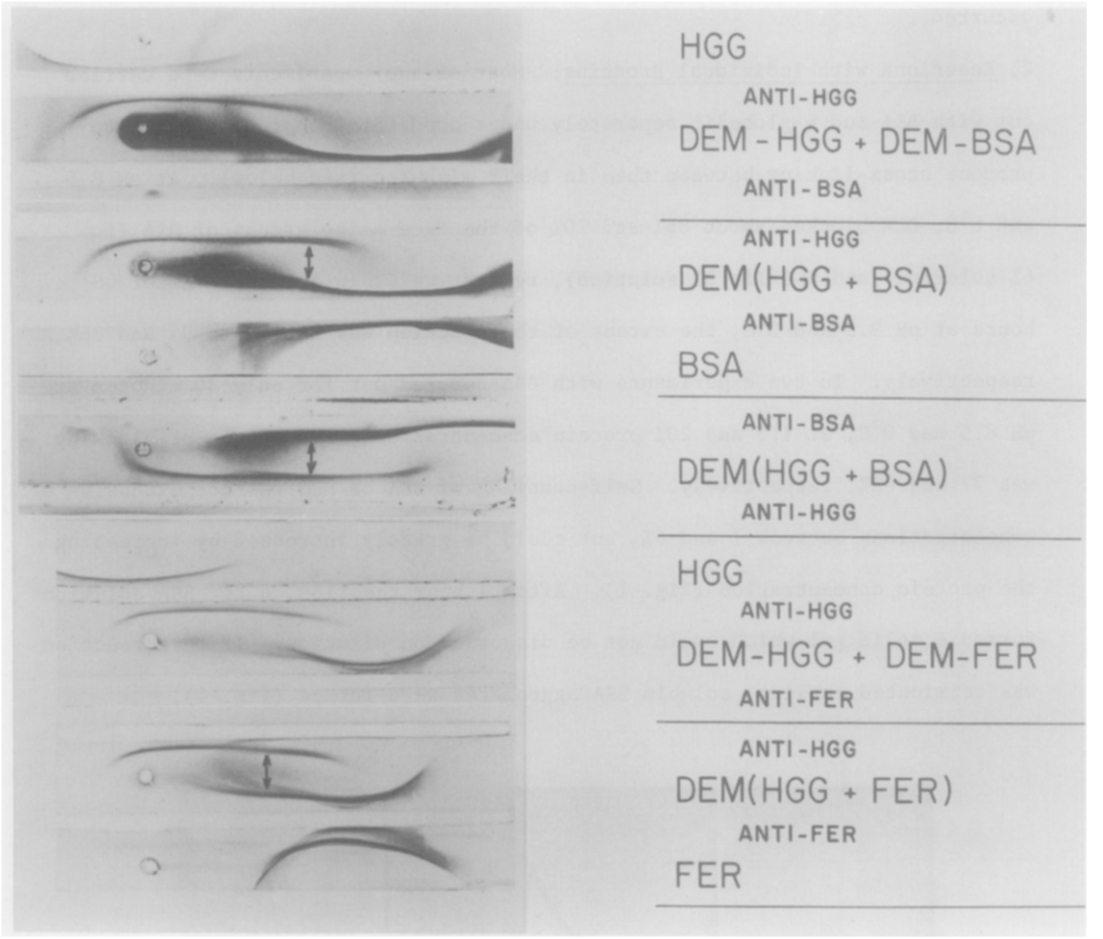


Figure 2. Immunoelectrophoresis patterns of DEM-modified and unmodified proteins developed with antisera directed to the latter. The proteins placed in each well, and the antisera in each trough, are indicated in the right hand legend. Cathodic electrophoretic migration is towards the right. The arrows indicate the arcs due to intermolecular conjugates. In the third slide from the top, the electrophoresis was prolonged so as to better resolve the conjugates.

The immunoelectrophoresis experiments (fig. 2) show that after extensive (80-90%) modification of their free amino groups both BSA and HGG: 1) retain their antigenic activities (i.e., their capacity to react with antibodies to the unmodified proteins) essentially undiminished; and 2) suffer no significant changes in their electrophoretic mobilities.

The amidination of 84% of the amino groups of anti-DNP antibodies with DEM resulted in no decrease in specific binding capacity for the hapten, DNPNS (Table I). Similar modification of RGG did not cause it to exhibit any non-specific binding capacity for DNPNS.

3) Cross-linking of BSA to HGG, and Ferritin to HGG. After some preliminary trials, most of the cross-linking experiments were carried out by the procedure given in the Methods section, at a concentration of 0.2 M DEM and of 6% BSA and

TABLE I

Hapten Binding of Anti-DNP Antibodies Before And After Modification With DEM*

Anti-DNP		DEM-Anti-DNP	
$c^{\ddagger}$ $\times 10^{5}$M	r/c^{**} $\times 10^{-5}$M^{-1}	$c^{\ddagger}$ $\times 10^{5}$M	r/c^{**} $\times 10^{-5}$M^{-1}
0.19	5.5	0.20	5.6
0.88	1.47	0.90	1.46
1.68	0.81	1.65	0.84
2.50	0.55	2.46	0.58
3.30	0.43	3.22	0.45

* As measured by titration with the dye hapten DNPNS (Metzger, et al., 1963). Concentrations of anti-DNP antibodies and DEM-modified anti-DNP were 1.24×10^{-5}M and 1.19×10^{-5}M, respectively.

‡ concentration of free hapten

** r is ratio of moles hapten bound per mole antibodies.

3% HGG, or 6% ferritin and 2% HGG, in the reaction mixture at pH 8.5 and 0°C for 24 hours. Upon immunoelectrophoresis, there was clear evidence (fig. 2) of the presence of some cross-linked reaction product. This component was identified (Schick and Singer, 1961) by virtue of 1) an electrophoretic mobility intermediate between the mobilities of the two uncoupled proteins, whereas no component with such mobility was visible with the same proteins which were separately reacted and then mixed; and 2) a capacity to react with antibodies specific for both of the original proteins in the mixture. Of the order of 10-20% of the total protein was cross-linked. No substantial increase in the extent of cross-linking was effected by carrying out the DEM reaction at pH 9.0 or 9.5 instead of 8.5. No systematic attempts were made, however, to improve the yield.

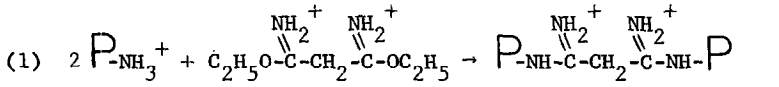
4) The Properties of Sheep Red Cells Modified with DEM. Unreacted sheep red cells maintained in the pH 8.5 buffer for 24 hours at 0°C, and red cells modified with ethyl acetimidate, both completely lysed after three washings with cold phosphate-buffered saline. The DEM-modified cells, however, did not lyse even after three weeks in this medium, nor after 1 hour in distilled water. Furthermore, they were completely resistant to immune hemolysis. Whether this resistance might have been due to a loss of capacity of the modified cells to bind specific anti-red cell antibodies could not readily be tested by immune hemagglutination experiments, because of the reversible clumping of the DEM-modified cells even in the absence of specific antisera.

DISCUSSION

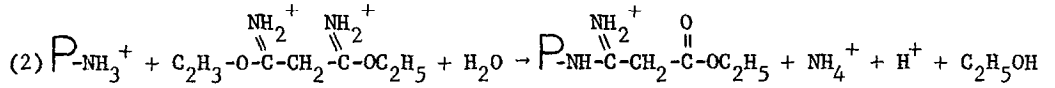
1) The Nature of the Reaction of DEM with Proteins. Monofunctional imidoesters have been shown to react specifically with the free amino groups of protein (Hunter and Ludwig, 1962; Wofsy and Singer, 1963), and it is assumed that the specificity of the DEM reaction is the same. In support of this, the electrophoretic mobilities of the DEM-modified proteins were essentially the same at pH 7.5 (fig. 2) as their unmodified counterparts. Had any

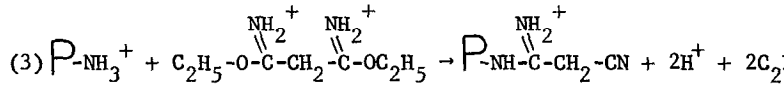
significant amount of stable product formed at histidyl, tyrosyl, or other residues, the net negative charge and cathodic electrophoretic mobility at pH 7.5 should have decreased.

Two classes of reaction products might form in the reaction of DEM with lysyl residues (or α-amino groups) of proteins. In one class, both imidoester groups of a DEM molecule might react to form a diamidine cross-link:



This cross-link might be intra- or inter-molecular. With extensively modified but only lightly aggregated proteins, the former links would be expected to predominate (Wold, 1961 a, b). Clearly, no change in net charge on the protein should occur with this class of reaction products. In the other class, one imidoester group of a DEM molecule might amidinate a lysyl residue, but the second might be hydrolyzed. Under ordinary conditions, the most likely hydrolysis product of an imidoester is an ordinary ester (equ. 2); a less likely product is the nitrile (equ. 3) (Roger and Neilson, 1961).





The instability of DEM is such (see Results section) that no unreacted, unhydrolyzed imidoester groups could remain on the protein. By either of these combinations of monofunctional and hydrolytic reactions, the net charge on the protein molecule should not be affected. We have no information about the relative extents of the two classes of reactions; only the total amount of protein NH_2-group modification was measured and was shown to be extensive under the reaction conditions studied.

2) Biological Activities of DEM-Modified Proteins. The immunoelectrophoresis experiments show that in spite of up to 90% modification of their NH_2-groups

by DEM the proteins BSA, HGG, and ferritin retain most if not all of their capacity to bind to antibodies specific for the respective unmodified proteins. The retention of antigenic activity is a sensitive criterion of the maintenance of protein conformation. The quantitative retention of antibody activity after the extensive modification of anti-DNP antibodies is similarly strong evidence that little or no conformational change was induced by reaction with DEM. These results may be compared with those obtained with acetamidinated proteins (Wofsy and Singer, 1963).

3) The Intermolecular Cross-Linking of Proteins by DEM. Individual proteins can be cross-linked and aggregated with DEM (fig. 1), or two different proteins can be cross-linked (fig. 2). Ferritin-antibody conjugates, cross-linked by diisocyanates (Singer, 1959; Schick and Singer, 1961) have been developed as specific stains for electron microscopy (Singer and Schick, 1961). Similar conjugates prepared with DEM (fig. 2) are currently being tested to determine their usefulness in electron microscopic studies. The capacity of DEM to produce intermolecular protein aggregates, without any substantial change in the biological or physical properties of the proteins involved, should be of considerable interest in a variety of biochemical studies.

4) The Modification of Red Blood Cells. Berg, Diamond, and Marfey (1965) have recently shown that reaction with the cross-linking agent 1, 5 difluoro-2,4-dinitrobenzene (Zahn and Stuerle, 1958) made red cells resistant to hypotonic lysis, whereas cells treated with the monofunctional reagent 1 fluoro- 2,4-dinitrobenzene could be lysed. Upon modification with these reagents, the constellation of charges on the red cell is probably altered radically, whereas this is not likely to be the case with DEM. DEM also rendered red cells resistant to hypotonic lysis; this resistance must be a result of cross-linking reactions, because the monofunctional imidoester did not produce it. Of further interest is the complete resistance to immune hemolysis which was conferred on red cells by reaction with DEM. These observations are under further investigation, along with structural and

physiological studies of the DEM-modified cells.

5) Bifunctional Imidoesters as Intramolecular Cross-Linking Agents.

An interesting feature of bifunctional imidoesters, $R'O\text{-}\overset{\overset{NH_2^+}{\|}}{C}\text{-}R\text{-}\overset{\overset{NH_2^+}{\|}}{C}\text{-}OR'$, is the fact that the size and geometry of the connecting structure R can be varied (McElvain and Schroeder, 1949). This family of reagents may therefore be especially useful in mapping out the distances between different lysine residues in the native conformation of a protein molecule (cf. Wold, 1961 a, b)

ACKNOWLEDGEMENTS

We are most grateful to Dr. Patricia S. Traylor for helpful advice and discussions. The immune hemolysis experiments were kindly carried out by Dr. Stanley E. Mills. This work was supported by U.S. Public Health Service grant No. AI-04255.

REFERENCES

Berg, H. C., Diamond, J. M., and Marfey, P. S., Science 150, 64 (1965).
Farah, F. S., Kern, M., and Eisen, H. N., J. Exptl. Med. 112, 1195 (1960).
Hunter, M. J. and Ludwig, M. L., J. Am. Chem. Soc. 84, 3491 (1962).
McElvain, S. M. and Schroeder, P., J. Am Chem. Soc. 71, 40 (1949).
Metzger, H., Wofsy, L., and Singer, S. J., Arch. Biochem. Biophys. 103, 206 (1963).
Roger, R. and Neilson, D. G., Chem. Rev. 61, 179 (1961).
Schick, A. F. and Singer, S. J., J. Biol. Chem. 236, 2477 (1961).
Singer, S. J., Nature 183, 1523 (1959).
Singer, S. J. and Schick, A. F., J. Biophys. Biochem. Cytol. 9, 519 (1961).
Wasserman, E. and Levine, L., J. Immunol. 87, 290 (1961).
Wofsy, L. and Singer, S. J., Biochemistry 2, 104 (1963).
Wold, F., J. Biol. Chem. 236, 106 (1961).
Wold, F., Biochim. Biophys. Acta 54, 604 (1961).
Zahn, H., and Meienhofer, J., Makromol. Chem. 26, 126 (1958).
Zahn, H., and Stuerle, H., Biochem. Z. 331, 29 (1958).