Reactions of a Lipid-soluble, Unsymmetrical, Cleavable, Cross-linking Reagent with Muscle Aldolase and Erythrocyte Membrane Proteins*

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The compound di-N-(2-nitro-4-azidophenyl)cystamine-S,S-dioxide (DNCO) has been synthesized and characterized. In a dark reaction this reagent will undergo efficient disulfide-sulffhydryl exchange with protein —SH groups, thus providing a covalently bound photoactivatable group. The cross-linked products obtained in applying this reagent to the soluble protein rabbit muscle aldolase have pointed out some of the analytical difficulties associated with mixtures of peptide products of identical molecular weight but different Stokes radii. When applied to erythrocyte membranes, a series of cross-linked complexes were produced which were similar to, but not identical with, those produced by water-soluble bisimidates, or CuI-orthophenanthroline (CuP)-catalyzed air oxidation of intrinsic —SH groups.

Low yields of complexes of Bands 2.2 and 2.3 with about 40,000 daltons of extra material were noted. Spots most easily explained as complexes of Band 3 with Bands 4.1, 4.2, 4.4, and 6 were noted. A similar spot for Band 5 could have been a Band 3 complex or a Band 5 trimer. As with the other reagents no clear evidence has yet been obtained for a complex of Band 5 with spectrin. A spot showing faster movement in the first dimension of Band 4.2 after treatment with DNCO (or CuP) suggests the formation of a disulfide link between distant parts of that chain through —SH groups that are very close together in the intact membrane. This behavior is similar to that seen even more clearly in the aldolase experiments.

The reagents most commonly used in chemical cross-linking studies have been highly water soluble compounds such as the bisalkylimidates (1-5). When applied to membranes, these reagents approach only those elements of the structure which are accessible to the aqueous environment. We thought it useful to add to the list of available reagents one which was relatively insoluble in water but quite soluble in organic phases. Such a reagent would carry out its cross-linking function actually within or very close to the lipid bilayer. Only a very limited number of accessible and chemically reactive groups are expected to occur within the lipid phase. The principal possibilities are the thioester function of methionine and an un-ionized sulphydryl group. Polar oxygen and nitrogen containing groups are expected to be either in contact with the internal or external aqueous phases or buried as hydrogen-bonded partners within the interior of the protein. Alkylation of thiethers can be carried out easily but leads to the very polar sulfonium cation which is likely to seriously perturb the protein and, by implication, the membrane in the immediate vicinity. Conversion of an un-ionized sulphydryl group to a disulfide produces no fixed charge nor any marked change in polarity. Perturbations should be limited to the steric effects of the attached reagent moiety.

To maximize the chances of forming a cross-link in the nonpolar environment of the bilayer, a highly reactive species such as a nitrene or carbene formed by photolysis (6, 7) should be at least part of the reagent. A symmetrical reagent with two photolyzable groups may have a relatively low probability of forming successful cross-links due to the many reactions open to the reactive groups. A successful insertion reaction would be required to occur twice in the same reagent molecule. An unsymmetrical reagent involving normal chemical modification for initial attachment and a photoactivatable group for the second step appeared attractive. One such compound is di-N-(2-nitro-4-azidophenyl)cystamine-S,S-dioxide (DNCO'). The disulfide of the parent symmetrical reagent is oxidized to the disulfide to enhance reactivity as a disulfide interchange reagent. The final cross-linked product retains the property of easy cleavability by reduction which simplifies subsequent analysis. The synthesis and tests of this compound as a cross-linking reagent are reported in this paper. The reaction scheme is shown in Fig. 1.

During this study reports of other noncleavable photochemical cross-linking reagents have appeared. Such compounds

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have been applied to enzyme immobilization (8), to the study of the active site of glyceraldehyde-3-phosphate dehydrogenase (9), and to the covalent attachment of hormones to receptor proteins (10). Multifunctional photoactivatable lipid-soluble reagents have been described by Klip and Gitler (11).

**MATERIALS AND METHODS**

The sources of chemicals and equipment were: 4-fluoro-3-nitroaniline, cysteamine dihydrochloride, acrylamide, N,N,N',N'-tetramethylthelenediamine, dichlorobenzotriol, N-ethylmaleimide, acrylamide, pyridined, dimethylsulfoxide, Aldrich; Minictron concentrator B-15, Amicon Corp.; Tris, rabbit muscle aldolase, phosphorylase a; catalase, ovalbumin, bovine serum albumin (Fraction V), Sigma; 5,5'-dithiobis(2-nitrobenzoic acid), dimethylsulfoxide, sodium dodecyl sulfate, Pierce; o-Phenanthroline, Matheson, Coleman and Bell; peracetic acid (40%) FMC Corp. All other chemicals were standard reagent grade. Distilled water was used throughout these experiments.

Elemental analyses were performed by Baron Consulting Co., New Haven, Conn. Infrared spectra were recorded on a Beckman IR4200 spectrophotometer with samples mixed in mineral oil. NMR spectra were recorded in parts per million (downfield of a tetramethylsilane internal standard) on a Bruker HX-270 instrument. Silica gel plates (Art. 5532/0001) were obtained from EM Laboratories. For thin layer silica gel chromatography, Solvent I (benzene:chloroform:acetic acid (1:1:0.9:0.4)); Solvent II (chloroform:pyridine (1:1)).

**Synthesis of DNC**—All azidothiol compounds were synthesized and handled in the dark or in very dim light. 4-Fluoro-3-nitrophenyl azide was synthesized according to the procedure of Fleet et al. (12).

Cysteamine dihydrochloride was recrystallized from ethanol before use. 1.95 g of cysteamine dihydrochloride (8.6 mmol) was dissolved in pyridine to (8.6 mmol) was dissolved in ethanol (200 ml) and 3.66 g of triethylenemine (36 mmol) was added. To this solution was added 7.00 g (39.6 mmol) of 4-fluoro-3-nitrophenyl azide pre-dissolved in a small amount of warm ethanol. This solution was left stirring in the dark for 24 h at 23°C. Large amounts of a red muddy precipitate formed. One thousand milliliters of cold H2O was added. The product was collected by filtration through Whatman No. 1 filter paper, dried at room temperature under reduced pressure, and recrystallized from benzene/hexane. Yield: 3.54 g (86%); m.p. 143-144°C (decomposition). Found: C 40.33, H 3.39, N 29.40, S 13.43, O 18.88. Calculated: C 40.18, H 3.17, N 29.32, S 13.41, O 18.88.

**Synthesis of DNCO**—Twice recrystallized DCS, 0.48 g (1 mmol), was dissolved in chloroform (400 ml) in a three-necked flask equipped with a calcium chloride drying tube and dropping funnel. The solution was cooled in an ice bath. Peracetic acid, 2 mmol diluted at 200 ml of chloroform, was added dropwise with continuous stirring. Reaction was continued for 4 to 5 h with ice cooling, and then 1 h at room temperature. The product was obtained by rotary evaporation of the solvent at room temperature, and recrystallization from pyridine as red needles. Yield: 0.15 g (30%); m.p. 127-128°C (decomposition). Found: C 37.70, H 3.37, N 27.45, S 12.33, O 18.53. Calculated: C 37.79, H 3.15, N 27.55, S 12.59, O 18.88.

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\text{C}_9\text{H}_8\text{O}_4\text{N}_2\text{S}_2
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**FIG. 2.** NMR peak positions and assignments for spectra of (a) DCS in pyridine-d₅, and (b) DNCO in dimethylsulfoxide. The peak for the amino proton is broad. The observed NMR peak positions and assignments are shown in Figure 2b. Cross-linking Procedures—DNCO was dissolved in pyridine to give a stock solution of 5 to 17 mg/ml. Aliquots of the solution were added rapidly to the samples agitated on a Vortex mixer or by stiring. Final pyridine concentration in the sample was less than 0.5% (v/v).

Crystalline aldolase was dissolved in 20 mm Tris/Cl buffer at pH 8.0 and dialyzed against the same buffer to remove last traces of ammonium sulfate. The concentration of aldolase was determined by absorbance at 280 nm, \(E_{280} = 0.91\). Aliquots of the stock solution of DNCO were added to tubes containing aldolase. After 60 min in the dark at room temperature, the samples were either kept in the dark for an additional 10 min or photolyzed for 10 min. Aliquots of concentrated SDS and NEM solutions were added to yield final concentrations of 2% and 0.5 mg/ml, respectively. For electrophoresis, the samples were usually concentrated to about 2 mg of protein/ml in a Minicon B-15 concentrator. When necessary the samples were stored frozen at -20°C; for analysis they were heated to 37°C for 30 min just prior to electrophoresis.

Cross-linking by cupric o-phenanthroline complex (Cu²⁺)-catalyzed oxidation, aliquots of a stock solution of Cu²⁺, 6.8 mM, were added to the sample to give a final concentration of 34 µM. After 60 min at room temperature, 0.5 mM EDTA, 2% SDS, and 0.5 mg/ml of NEM were added to quench the reaction.

Human erythrocyte membrane ghosts were prepared according to the procedure of Fairbanks et al. (14) and were used the same day. NEM-treated ghosts were prepared as described in Ref. 15. Protein concentration was estimated by the method of Lowry et al. (16) in the presence of 1% SDS.

Standard Buffer A consisted of 50 mm Tris, 1 mm MgCl₂, adjusted to pH 8.0.

Ghosts or NEM-treated ghosts were suspended in Buffer A at a concentration of 0.5 mg of protein/ml. Aliquots of the DNCO stock solution were added to the membrane suspension with stirring in the dark at room temperature. The samples were left in the dark for 30 min.

Centrifugation at 27,000 × g for 30 s removed unreacted solid reagent, if any. The membranes were centrifuged out at 27,000 × g for 20 min at 4°C, and then resuspended in half of their original volume.

The asterisk indicates the peak that overlaps with the solvent peak, (d) identifies doublet peaks, (q) quartets, and (m) multiplets.
A.) The membrane sample was divided into two parts. One was kept in the dark, the other was photolyzed at room temperature for 10 min. NEM was then added to a final concentration of 0.3 to 1.0 mg/ml. The membranes were pelleted by centrifugation at 27,000 × g for 20 min, followed by solution in gel buffer (14) containing 4% SDS and 4% NEM of protein/ml. These samples were held at 37°C for 1 h before electrophoresis.

Photolysis—Photolysis was carried out with the sample in a small beaker or Petri dish and a liquid thickness of less than 2 mm. The front surface of a GE photolamp EAL 500 W was placed about 19 cm above the sample layer with 8 cm of ice water in between as a thermal filter. The rise in the sample temperature was less than 4°C. The normal photolysis time was 10 min when, as judged by cross-linking patterns and absorption at λmax, of DNCO, loss of the azide was complete.

Titration of Unreacted Sulffhydryl Groups—The total sulfhydryl content of the enzyme or membrane samples and their DNCO-modified derivatives was estimated by monitoring spectrophotometrically their reaction with DTNB (17). The following stock solutions were prepared before assay: (a) 10 mm DTNB in 0.1 M phosphate buffer, pH 7.0; (b) 1% SDS, 0.2 mm EDTA, 0.1 M Tris/Cl at pH 8.0; (c) sample at a concentration of 0.5 mg of protein/ml of buffered solution.

In the reference cuvette was placed 0.9 ml of b; in the sample cuvette, 0.1 ml of a, 0.8 ml of b. Reaction was initiated by the addition of 0.45 ml of c to both cuvettes. Maximal absorbance at 412 nm was reached after 5 to 10 min. After correcting for the absorbance of DTNB, thiol contents were calculated assuming an E412, nm of 1.36 × 10⁴ M⁻¹ cm⁻¹ for the 3-carboxy-4-nitrothiophenolate anion (16). The molecular weight of aldolase tetramer was taken as 160,000.

A 20- to 30-fold molar excess of DTNB over any DNCO in the sample was used to minimize any interference in the assay. The maximum error from this source was never greater than 1% and the precision of the DTNB titration was usually ±5%.

SDS-Polyacrylamide Gel Electrophoresis—The procedure of Fairbanks et al. (14) was used to prepare slab gels (15 × 15 × 0.3 cm) with the following specific concentrations: SDS 0.2% (w/v), acrylamide 5.25% (w/v), N,N'-methylenebisacrylamide 0.1% (w/v). Thiol groups were omitted when analyzing the cross-linked samples. The slab gel was run at 40 to 80 mA/slab overnight, stained with Coomasie blue, and destained as described in Ref. 15. For testing reversibility of cross-linking in one-dimensional gels, samples in SDS solutions were reduced by adding an equal volume of 1 M dithiothreitol solution in 1 M Tris/Cl buffer at pH 7.5. The solution was kept at 37°C for 1 to 3 h before electrophoresis.

Two-dimensional gel electrophoresis, molecular weight calibrations, and identification of band of origin for off-diagonal spots were carried out as described in Ref. 15.

RESULTS AND DISCUSSION

Of the eight sulfhydryl groups per monomer unit of aldolase (19, 20), Steinman and Richards (17) found that a series of disulfide monosulfides reacted with a maximum of four of these groups in the undenatured enzyme. The extent of reaction of DNCO is shown in Fig. 3a. The maximum modification actually measured was 2.5, although the saturation level may be larger than any of those used in the earlier study. Thus steric or reactivity differences (or both) may play a role in the one—SH group difference in extent of modification.

When aldolase was pretreated with CuP, only one additional —SH group per monomer could be modified by DNCO (Fig. 3a). DNCO apparently reacts with the same two —SH groups as does CuP. There is one additional group available to DNCO which is unaffected by CuP. The fourth —SH group is unreactive towards both reagents.

It is known that cysteine 72 and cysteine 336 must be close together in the three-dimensional structure since a disulfide bond is readily formed between them on CuP-catalyzed oxidation (19–21). It also seems this cross-link is formed to a small extent by a dark reaction with DNCO. The initial disulfide formed between the reagent and one member of the pair probably undergoes a disulfide interchange reaction with the other peptide —SH and expulsion of the aryl azide reagent. In SDS such circular peptides will have a lower Stokes radius than a linear peptide of the same molecular weight and would thus be expected to move faster in an acrylamide gel system.

This behavior is shown in Fig. 4a. Samples 5, 7, and 9, where a small amount of material moving faster than the bulk of the monomer is clearly visible. If not recognized, this material might be considered a different peptide with an apparent molecular weight about 4000 less than the aldolase monomer. A comparable effect has been seen in lysozyme (22).

The arrangement of this particular pair of sulfhydryl residues is peculiarly favorable for a disulfide interchange to take
the photolysis products are seen. The yield appears to be whose extent of modification is shown in Fig. 3a. Even in the SH per monomer (Sample 5). The marked increase in the fast moving bands comparable to those seen for the monomer. change process. The dimer region is also split into slow and to be substantially more than through the dark disulfide inter- radii would again be expected to be significantly different even though the molecular weights are identical.

gel loading. Diffuse trimer and tetramer bands can now be even higher DNCO concentrations and with somewhat higher that did not enter the top of the gel. In the CUP-catalyzed oxidation shown in Sample 11, the cyclic monomer band is

Fig. 4. Cross-linking of aldolase with DNCO and CuP. a. Samples 1 to 10 were prepared in pairs. In each pair, the first is a dark control; the second is a photolized sample indicated by an arrow. The aldolase concentration was 0.5 mg/ml in all samples. Slots 1 and 2, native aldolase; Slots 3 and 4, with pyridine 0.5% v/v; Slots 5 and 6, with DNCO (42.5 µg/ml); Slots 7 and 8, with DNCO (85 µg/ml); Slots 9 and 10, with DNCO (85 µg/ml) after 1 h in the dark, 4 volumes of 20 mm Tris/Cl, pH 8.0, was added just prior to the photolysis step; Slot II, with 34 µM CuP, air oxidation; Slot 12, protein standards. Left: (from above) phosphorylase a (M, = 92,000), catalase (M, = 61,000), reduced aldolase (M, = 40,000). Right: ovalbumin (M, = 45,000) cross-linked with dimethyl suberimide as described in Ref. 38. 6, samples from a after reduction with dithiothreitol (DTT).

place. In general, this interchange reaction is expected to play a relatively minor role. The reactivity of the disulfide group itself is much less than the mono or dioxide derivatives (17, 23). The high concentrations of free—SH groups necessary for effective interchange will not normally be present in a stable oligomer and only in very concentrated solutions will the neighboring oligomeric units provide the necessary concentration through collision complexes. For nearest neighbor analysis the interchange reaction, which results in a cross-link similar to that of the intended reagent reaction, will not alter the conclusions based on the product analysis.

After photolysis, the DNCO-modified samples all show both a marked increase in the amount of the fast moving band as well as the appearance of multimeric products, especially the dimer. In Fig. 3b are shown the gel patterns of the samples whose extent of modification is shown in Fig. 3a. Even in the sample with only 0.3 reagent molecule per monomer, traces of the photolysis products are seen. The yield appears to be essentially unchanged with modification beyond about 1.5 —SH per monomer (Sample 5). The marked increase in the fast moving band indicates the formation of the cyclized peptide by the photolysis reaction. The yield by this route appears to be substantially more than through the dark disulfide interchange process. The dimer region is also split into slow and fast moving bands comparable to those seen for the monomer. These presumably represent linear monomer—linear monomer and linear monomer—cyclic monomer complexes whose Stokes radii would again be expected to be significantly different even though the molecular weights are identical.

In Fig. 4a are shown some additional samples prepared at even higher DNCO concentrations and with somewhat higher gel loading. Diffuse trimer and tetramer bands can now be seen and, particularly in the case of Sample 8, some material that did not enter the top of the gel. In the CuP-catalyzed oxidation shown in Sample 11, the cyclic monomer band is formed very efficiently while the dimer band is weak and no higher oligomers are visible. It is thus probable that most of the dimer and higher bands in Samples 6 and 8 arise from intratetramer rather than intertetramer cross-linking reactions. This is confirmed by Sample 10 which was identical to Sample 8 except that it was diluted by a factor of 5 prior to photolysis. The product distributions in 8 and 10 are indistinguishable. That higher concentrations of trimer and tetramer bands relative to the dimer were not seen may be due either to the particular spatial distribution of the SH groups modified by DNCO or to nonidentical arrangement of the four subunits as suggested by other cross-linking studies and by x-ray diffraction data (24, 25).

One notes in Fig. 4a that the cross-linking with CuP to yield the fast monomer species is almost complete while in the DNCO reaction a substantial amount of normal monomer remains. The probable explanation lies in solvolysis, hydrogen abstraction, or other fates of the nitrene which do not lead to covalent cross-links. The probability of these side reactions may be quite high and will depend on the details of the environment of the nitrene. For proteins with only a small number of reagent molecules attached (a maximum of about 3 per monomer for aldolase), the predicted fraction of uncross-linked products would be (1 - p)n where p is the mean probability of forming a cross-link for a single nitrene and n is the maximum number of groups potentially able to produce cross-links. If p = 1/2 and n = 3, then about 30% of the material would remain monomeric at the end of the reaction. On the other hand, if amino groups had been modified there would have been many more reagent molecules attached and although the individual probability of cross-linking for each group might be the same, the fraction of material cross-linked would approach unity. Thus in the above example, if n = 10 and p is still 1/2, the fraction of remaining monomer would be less than 2%. This is based on the fact that only a single cross-link is required to produce a product with modified mobility in the analytical gels.

In Fig. 4b are shown the Fig. 4a samples after reduction of the disulfide groups. Essentially only the linear monomer peptide is seen. All the fast moving bands and the higher oligomers have been cleaved. There is only a residual trace of dimer left. The origin of this material is not known but it is likely to be cross-linked by groups not involving a disulfide function. The most obvious possibility would be radical recombination in minor photolysis paths involving hydrogen abstraction rather than the hoped for insertion reaction.

With the DTNB assay, under conditions where the membrane proteins are fully denatured, the total —SH content of erythrocyte ghosts is about 50 nmol per mg of protein (26). With intact ghosts about 70% of these groups react with N-ethylmaleimide, and about 30% with DNCO (Fig. 5). With NEM-treated ghosts none of the residual groups are available for reaction with DNCO as estimated by the DTNB titration, nor do the gel patterns of photolysed samples of such ghosts show any evidence of cross-linked products.

The gel patterns of ghosts cross-linked with increasing concentrations of DNCO are shown in Fig. 6. Photolysis in the presence or absence of pyridine produces only traces of cross-linked products, presumably through air oxidation of —SH groups. With as little as 7 nmol of DNCO per mg of protein easily seen cross-linked complexes appear. On reduction of any of the samples with dithiothreitol, the original band pattern is almost completely restored.

In two-dimensional gels of ghosts reacted with DNCO in the
In the dark a small amount of material derived from a spectrin–Band 4.5 complex was always seen. This same complex is produced in high yield with CuP (15). On photooxidation of the DNCO-treated sample, the 4.5 complex shifts to a very high molecular weight form, reflecting the rapid polymerization of spectrin itself. In the ghosts there would appear to be at least two closely opposed —SH groups, one on Band 4.5 and one on spectrin in an environment resembling that found for residues 72 and 336 in aldolase. As in the soluble enzyme, a two-step disulfide interchange reaction with DNCO in the dark occurs sufficiently often to produce a detectable yield of the cross-linked product.

Some typical two-dimensional gels of photolyzed reaction products are shown in Fig. 7. Usually 3.2% acrylamide gels were used in the first dimension as described in Ref. 15. In an attempt to get better resolution in the high molecular weight region, some gels were run at 2.8% in the first dimension with longer running times in both dimensions. While this procedure did improve the separation and permitted clearer visualization of certain spots, the long streaks of Bands 1 and 2 were still essentially continuous. This may be due to the association of spectrin with a large number of low molecular weight components which produces such an array of molecular weights in roughly comparable amounts that no apparent resolution is obtained in this system. The band spreading expected for cross-linked preparations separated by techniques sensitive to Stokes radius would be expected to contribute to this problem (2). The very high molecular weight material that does not enter the 3.2% gel and barely enters the 2.8% gel.

**Fig. 5.** Modification by DNCO of —SH groups in native ghosts and NEM-treated ghosts. Samples in Buffer A at a concentration of 1 to 2 mg of protein per ml were treated with DNCO in the dark with stirring. After 30 min at room temperature, aliquots were withdrawn for assay of the remaining —SH groups: ○—○, ghosts treated only with DNCO; ▲—▲, ghosts pretreated with NEM and then exposed to the indicated concentration of DNCO.

**Fig. 6.** One-dimensional gels of erythrocyte ghost proteins. All samples were paired, dark control on the left, photolyzed sample on the right. Slots 1 and 2, untreated membranes; Slots 3 and 4, membranes exposed to the amount of pyridine normally added with reagent; Slots 5 through 12, samples with the indicated amount of reagent. The four samples at the right show the effect of dithiothreitol (DTT) reduction. Acrylamide concentration: 3.2%.
FIG. 7. Two-dimensional gels of erythrocyte membrane proteins after DNCO treatment. a, acrylamide concentration, 3.2% in first dimension (horizontal), and 5.2% in second dimension (vertical), DNCO (7.0 nmol/mg of protein); b, same as a except DNCO (28 nmol/mg of protein); c, same as a except DNCO (56 nmol/mg of protein); d, acrylamide concentration, 2.8% in first dimension, and 5.2% in second dimension, longer running time in both dimensions, only top of gel shown, and DNCO (14 nmol/mg of protein); e, same as d except DNCO (28 nmol/mg of protein) and longer running time in second dimension; f, bottom part of heavily loaded gel equivalent to b.

is seen with DNCO cross-linking as with all other cross-linking agents so far tried. This material contains essentially all of the membrane protein components and is most easily explained by association of these components with polymerized spectrin (15).

No one sample or single gel can show all of the components. A summary of a number of gel patterns obtained under differing cross-linking conditions with DNCO is shown in Fig. 8. Many aspects of the pattern are similar to those reported earlier for CuP or the bisimidate, DTBP, cross-linking; however, there are differences. The spectrin oligomers and the Band 3 dimer appear most rapidly and in the largest relative amounts, as with the other reagents. Low yields of complexes of Bands 2.2 and 2.3 with about 40,000 daltons of extra material were noted. These have not been reported with either CuP or DTBP. While some complexes for Bands 2.1 to 2.3 are seen in the 350,000 to 400,000 region, many of the higher molecular weight complexes reported for 2.4 and 2.5 with CuP were not detected with DNCO. In the low molecular weight region DNCO produced more complexes than CuP. A series most easily explained as 1:1 complexes with Band 3 material are seen for Bands 4.1, 4.2, 4.4, 5, and 6, although the Band 5 spot could equally well be a trimer of Band 5 itself. The extended spots for Bands 4.2, 4.5, and 6 at 200,000 to 300,000 daltons are probably a reflection of complexes of these materials with spectrin, as noted earlier. As in the earlier studies, no clear evidence was obtained for the association of Band 5 with spectrin.

After the cross-linking reaction the monomer band of 4.2 moves slightly faster in the first dimension (Fig. 6, Slot 12), than the uncross-linked material (Fig. 6, Slot 11). In the two-dimensional gels this material appears to the right of the diagonal monomer line, Fig. 7. This behavior is also noted with CuP (Ref. 15, Fig. 3, b and e), and suggests that an
such as nitrenes. In significant amounts after prolonged reaction with reagents is most easily explained by the existence of stable, time-independent complexes in the membrane. On the other hand, CUP and DNCO have similar size and hydrophobicity. Both require accessible -SH groups for cross-linking, but the reactivity of the nitrene is unknown.

The curved dashed line indicates the locus of expected positions for spots derived from complexes of the appropriate monomer and an additional $M_r = 90,000$ peptide. The gels do not separate effectively outside of the region indicated by the short dash lines. The spots shown on the left are derived from material which effectively did not enter the first dimension gels.

**Fig. 8.** Schematic summary of erythrocyte cross-linking results with DNCO. Top portion refers to upper abscissa and the more dilute first dimension gels. The bottom portion refers to the lower abscissa. The curved dashed line indicates the locus of expected positions for spots derived from complexes of the appropriate monomer and an additional $M_r = 90,000$ peptide. The gels do not separate effectively outside of the region indicated by the short dash lines. The spots shown on the left are derived from material which effectively did not enter the first dimension gels.

intrapeptide cross-link between distant parts of the chain has been formed comparable to the aldolase example discussed earlier.

Sulfhydryl groups in erythrocyte membranes have been shown to play a role in hydrophobic bonding of the protein components (27, 28). Modification with a negatively charged reagent, p-chloromercuribenzenesulfonate, extracts up to 40% of the protein. NEM does not lead to specific loss of membrane proteins but produces fragmentation and vesiculation of the membrane. These perturbations do not appear to occur in DNCO-treated ghosts. Probably the introduction of the very nonpolar moiety of DNCO does not weaken the hydrophobic bonding, and the disulfide bond produced is uncharged, as are the starting -SH groups near neutral pH.

As a nonpolar reagent DNCO should insert cross-links in the membrane in an area quite different from that affected by water-soluble systems. Its different characteristics make it complementary to the many other types of bifunctional reagents (2). DNCO concentrations on the order of 5 to $50 \mu g/ml$ are frequently adequate to give efficient cross-linking. Because of the two-step nature of the process, the effects, if any, of -SH group modification in the absence of cross-linking can be established. The subsequent effect of the cross-link per se can then be investigated separately. The initial attachment step is highly selective for -SH groups and thus, in general, will produce limited modification. The intrinsic reaction rate of the second step is controlled by the chemistry of the particular aryl nitrene, but the effective rate and extent of cross-linking can be controlled by the intensity and duration of the photolyzing radiation. The nitrene reaction is essentially pH and temperature independent. Therefore, although the initial SS exchange step requires neutral pH and reasonable temperature, the cross-linking can be carried out at liquid nitrogen temperatures if required. Since only a limited number of reagent molecules are normally attached in Step 1, and these few are located on very specific groups, the cross-linking reaction may be very sensitive to subunit conformation as well as to the oligomeric structure of the complex. A possible example of the latter and the application of DNCO to the calcium pump protein of sarcoplasmic reticulum will be described in a future communication.

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aldehyde-3-phosphate dehydrogenase (30-33). Recent studies show that Band 6 was co-purified with dimer of Band 3 and it can be dissociated from or reassocicated with Band 3 (34). Although neither CUP nor DTBP produced detectable cross-linking between Bands 3 and 6, DNCO did. Both monomers and dimers of Band 6 were found cross-linked to Band 3. The high content of cholesterol and of saturated fatty acids suggest a low fluidity of the lipid matrix in erythrocyte membranes. Recent studies using fluorescein-isothiocyanate labeling give an upper limit for the translational protein diffusion coefficient of $3 \times 10^{-12} \text{cm}^2/\text{s}$ at 90-23°C (35). The lifetime of an aryl nitrene is generally in the millisecond range (36, 37). Taking these two values, the average distance a protein can diffuse in 1 ms is less than 11 Å. Hence, it is probably true that DNCO cross-links reflect only stable protein complexes in erythrocyte membranes. A general discussion of the difficulties in interpreting negative cross-linking results and in distinguishing between stable and collision complexes has been given elsewhere (2). Although specifically intended for cross-linking studies in a nonaqueous environment, DNCO can be used effectively in water-soluble systems. Its different characteristics make it complementary to the many other types of bifunctional reagents (2). DNCO concentrations on the order of 5 to $50 \mu g/ml$ are frequently adequate to give efficient cross-linking. Because of the two-step nature of the process, the effects, if any, of -SH group modification in the absence of cross-linking can be established. The subsequent effect of the cross-link per se can then be investigated separately. The initial attachment step is highly selective for -SH groups and thus, in general, will produce limited modification. The intrinsic reaction rate of the second step is controlled by the chemistry of the particular aryl nitrene, but the effective rate and extent of cross-linking can be controlled by the intensity and duration of the photolyzing radiation. The nitrene reaction is essentially pH and temperature independent. Therefore, although the initial SS exchange step requires neutral pH and reasonable temperature, the cross-linking can be carried out at liquid nitrogen temperatures if required. Since only a limited number of reagent molecules are normally attached in Step 1, and these few are located on very specific groups, the cross-linking reaction may be very sensitive to subunit conformation as well as to the oligomeric structure of the complex. A possible example of the latter and the application of DNCO to the calcium pump protein of sarcoplasmic reticulum will be described in a future communication.

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