Nevertheless, the value of polarization (P) found for PMP appears to differ significantly from the value P equal to 0.06 reported by Churchich (*10*), who employed a double-beam, null-point, polarization photometer also said to measure polarizations to ± 0.002 (*18*).

In addition to data on the emission spectrum and on quantum yield, the fluorescence polarization spectrum has been advocated by Feofilov (*19*) as an independent parameter characteristic of the fluorescence of a given compound. The polarization of PMP fluorescence in 90 percent glycerol was measured as a function of exciting wavelength at 8°C (Fig. 4). The excitation spectrum shows two separated bands having peaks at ~250 mμ and 325 mμ. The polarizations in the regions corresponding to these bands are vastly different; and there is virtually no overlap between the two bands, as indicated by the very sharp transition from a highly positively polarized ($P = +0.39$) to a negatively polarized region ($P = -0.23$) in the excitation spectrum. According to accepted interpretations of such data (*19*), the direction of the absorption oscillators responsible for these two bands are probably mutually perpendicular, and the long wavelength electronic transition for absorption has a dipole moment parallel to that for emission.

It is not clear why there should be such a wide discrepancy between my results for the quantum yield, 0.14, of PMP and that reported by Churchich (*9, 10*), which is about four times the value reported here. Only part of this discrepancy can be accounted for by his use of 1-dimethylaminonaphthalene-5-sulfonate as a fluorescence standard having a quantum yield of 0.53 (*9*). This quantum yield, reported by Weber and Teale (*20*), is much higher than that found in other laboratories (*5, 21*).

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Disulfide-Bond Cleavage and Formation in Proteins

Abstract. *Disulfide bonds can be cleaved at an alkaline* p*H by treating a protein with excess of a reagent disulfide in the presence of catalytic amounts of thiol. The cleavage products are stable and can be isolated; they contain the mixed disulfide between the reagent and the exposed thiol groups of the protein. The extent of cleavage is readily controlled by the* p*H of the reaction, temperature, and the addition of urea. Disulfide bonds cleaved by the reaction can be re-formed by exposing the mixed disulfide of the protein to catalytic amounts of thiol. Specific side chains can be added on to the thiol groups in native proteins by treatment with a reagent disulfide alone.*

Thiols have been used extensively for the specific cleavage of disulfide bonds in proteins by the disulfide interchange reaction at an alkaline *p*H (*1*). The resulting protein cleavage products have thiol groups which are sensitive to oxidation. When the re-formation of disulfide bonds by air oxidation is under investigation (*2*) this sensitivity is desirable, but it is disadvantageous during physical and chemical studies of the cleavage products. For such studies the exposed protein thiol groups are therefore often protected from undesirable oxidation by alkylation with iodoacetamide or *N*-ethylmaleimide. The alkyl groups cannot, however, be removed subsequently. I have found that under suitable conditions the disulfide interchange reaction can be used for the cleavage of protein disulfide bonds, giving in a single-step process a stable product, insensitive to oxidation, which can be isolated. The product can nevertheless be used in experiments in which disulfide bonds are to be re-formed.

The rationale of the procedure is illustrated by the following equations. Equation 1 shows the first reaction between an ionized thiol (R-S$^-$) and a protein disulfide bond.

$$\text{Protein-S-S-Protein}' + \text{R-S}^- \rightleftarrows \text{Protein-S}^- + \text{R-S-S-Protein}' \quad (1)$$

In the presence of excess thiol the reaction continues and the protein thiols (Protein-S$^-$ and S$^-$-Protein′) are the chief products.

$$\text{R-S}^- + \text{R-S-S-Protein}' \rightleftarrows \text{S}^-\text{-Protein}' + \text{R-S-S-R} \quad (2)$$

In the presence of an excess of the reagent disulfide (R-S-S-R), the reaction in Eq. 2 is replaced by the reaction in Eq. 3.

$$\text{Protein-S}^- + \text{R-S-S-R} \rightleftarrows \text{Protein-S-S-R} + \text{R-S}^- \quad (3)$$

Under these conditions the thiol is not consumed in the overall reaction but serves as a catalyst. The overall reaction, which yields as the products the mixed disulfides between the protein and the reagent (Protein-S-S-R and R-S-S-Protein′), is then more suitably written

$$\text{Protein-S-S-Protein}' + \text{R-S-S-R} \overset{\text{R-S}^-}{\rightleftarrows} \text{Protein-S-S-R} + \text{R-S-S-Protein}' \quad (4)$$

The protein cleavage products are stable, with their thiol groups fully protected from oxidation. Yet, since all the reactions are reversible, the protective groups can at any time be removed or the disulfide bonds may be re-formed by exposing the products to a suitable concentration of the thiol in the absence of the reagent disulfide.

I must emphasize that the disulfide

interchange reaction at an alkaline *p*H in the presence of catalytic amounts of a thiol is well known and has been studied in the past by other workers (*3*). However, the usefulness of the reaction in the context discussed now appears to have been overlooked or neglected.

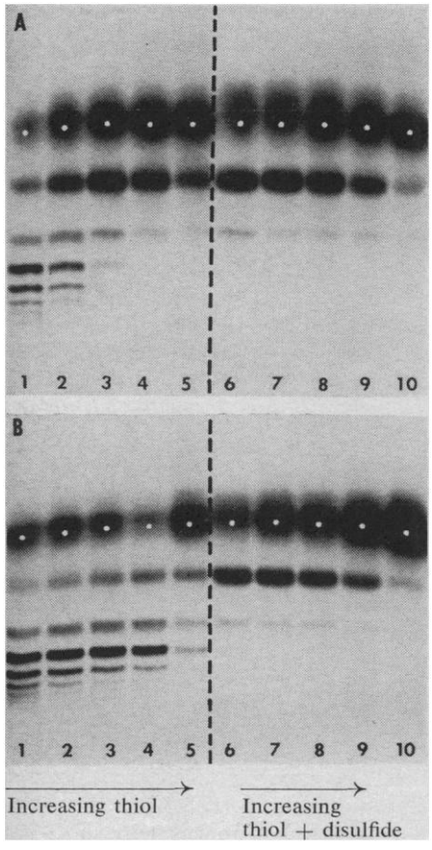

Fig. 1. Reversible and irreversible depolymerization of haptoglobin by the disulfide interchange reaction. (*A*) Benzidine-stained electrophoresis patterns of haptoglobin of type 2-2 after incubation for 1 hour at 37°C with increasing amounts of mercaptoethanol: (Samples 1 to 5) ME alone; (samples 6 to 10) ME plus its disulfide, diethanoldisulfide. Hemoglobin was added to the samples prior to electrophoresis. The zones corresponding to unbound hemoglobin are indicated by white dots. All other zones are complexes of hemoglobin and haptoglobin. (*B*) Patterns obtained after storage of the same samples for 24 hours at room temperature. Note the marked changes after storage in the samples (1 to 5) incubated with ME alone, but the absence of similar changes in the samples (6 to 10) incubated with ME plus diethanoldisulfide (DEDS). The haptoglobin samples contained borate-EDTA (*p*H 8.7) buffer plus: (sample 1) 2.5 m*M* ME; (sample 2) 5 m*M* ME; (sample 3) 10 m*M* ME; (sample 4) 20 m*M* ME; (sample 5) 40 m*M* ME; (sample 6) 100 m*M* DEDS + 2.5 m*M* ME; (sample 7) 100 m*M* DEDS + 5 m*M* ME; (sample 8) 100 m*M* DEDS + 10 m*M* ME; (sample 9) 100 m*M* DEDS + 20 m*M* ME; (sample 10) 100 m*M* DEDS + 40 m*M* ME.

A different use for the reaction of a protein with a reagent disulfide is apparent from Eq. 3, which indicates that many useful derivatives of proteins having accessible thiol groups can be obtained by treatment of the protein with a reagent disulfide alone (that is, in the absence of the corresponding thiol). This reaction has been used by Ellman (*4*) for the estimation of thiol groups in protein, and by Kanarek *et al.* (*5*) for the preparation of the mixed disulfide between reduced lysozyme and cysteine.

The following experiments illustrate the application of the reaction described by Eq. 4 to a specific problem. Haptoglobin (Hp) is a hemoglobin-binding plasma protein which shows genetically controlled variability in humans (*6*). One of the variant forms of haptoglobin (Hp2-2) contains a series of polymers (*7*). Conditions were sought to depolymerize the protein without destroying its hemoglobin-binding properties and to show that the polymers could be reformed nonenzymatically in vitro.

The first experiment was designed to establish conditions under which depolymerization could be observed in the presence of the thiol alone. Purified Hp2-2 was subjected to electrophoresis in borate-ethylenediaminetetraacetate (EDTA) buffered starch gels (*p*H approximately 8.5) containing 2-mercaptoethanol (ME) at several concentrations up to 0.01*M*. At room temperature the protein polymers were stable at all the concentrations of ME tested, but at 37°C the haptoglobin was completely depolymerized at concentrations greater than 0.007*M*.

Tests were then made of the effects of incubating haptoglobin at several temperatures (0° to 45°C) and *p*H's (8.0 to 9.5) in the presence of different concentrations (0 to 1*M*) of ME and its disulfide, diethanoldisulfide (*8*), (0 to 0.3*M*); the products of the reaction were studied by electrophoresis in starch gels. The results of these tests are most readily described in terms of the effect of the variables on the initial cleavage reaction (Eq. 1) and on the subsequent pathway of the reaction to either the protein thiol (Eq. 2) or the mixed disulfide (Eq. 3). The initial cleavage (Eq. 1) is dependent on the presence of ME; no cleavage is observed with diethanoldisulfide alone. The rate and extent of the cleavage increase with the concentration of ME, its degree of ionization (determined by the *p*H), and the reaction temperature. Addition of urea also increases the initial cleavage. Under severe conditions (high ME, or high *p*H, or with urea) the cleavage is extensive and causes the dissociation of the protein into its constituent polypeptide chains with loss of its hemoglobin-binding properties (*9*).

The ratio of the reagent disulfide to the thiol determines whether the cleavage product is obtained as the thiol (Eq. 2) or as the mixed disulfide (Eq. 3). I have found that diethanoldisulfide acts as a catalyst for the air oxidation of ME, as judged by a progressive decrease in the iodine-reducing capacity of the reaction mixture; consequently the ratio of diethanoldisulfide to ME increases during the reaction if it is carried out aerobically. This effect makes it easier to obtain and eventually isolate the mixed disulfide uncontaminated by the protein thiol. Under conditions which permit air oxidation of the ME, any initial ratio of diethanoldisulfide to ME of greater than five appeared to be sufficient to ensure complete mixed disulfide formation, although a ratio of 25 was finally selected. A temperature of 37°C and a *p*H of approximately 8.7 were satisfactory in ensuring that the initial cleavage reaction (Eq. 1) did not destroy the hemoglobin-binding properties of the haptoglobin. At lower *p*H's and temperatures the overall cleavage reaction was too slow or not sufficiently extensive; at higher *p*H's and temperatures the cleavage reaction was too extensive, and the product was no longer hemoglobin-binding.

The final selection of the conditions was made as a result of the experiment illustrated in Fig. 1 which also shows the stability of the protein-cleavage product, the mixed disulfide. The Hp2-2 was incubated at 37°C in borate-EDTA buffer (0.1*M* boric acid, 0.004*M* EDTA, 0.04*M* NaOH) of *p*H 8.7, with 0.0025*M*, 0.005*M*, 0.01*M*, 0.02*M* and 0.04*M* ME in the presence and absence of 0.1*M* diethanoldisulfide. After 1 hour, hemoglobin was added to a portion of each sample and electrophoresis was carried out at room temperature for approximately 20 hours at 4 volt/cm in a starch gel containing 0.025*M* boric

acid, 0.001*M* EDTA, and 0.012*M* NaOH. The results, after benzidine staining, are shown in Fig. 1*A*. Essentially complete depolymerization was observed in the presence of 0.1*M* diethanoldisulfide at all concentrations of ME greater than or equal to 0.005*M* (samples 7, 8, and 9), but at the highest concentration (0.04*M*) the hemoglobin-binding capacity of the product was partially lost (sample 10). In the absence of the diethanoldisulfide, depolymerization was not complete at concentrations of ME less than 0.02*M* (samples 1, 2, and 3), a concentration greater than that (0.007*M*) noted above because partial repolymerization can occur when the electrophoresis is carried out in the absence of ME. After storage of the samples for 24 hours at room temperature, hemoglobin was added to a second portion, and electrophoresis was again carried out. The results (Fig. 1*B*) establish that all the samples which had not been treated with the disulfide repolymerized extensively (samples 1 to 5), whereas the treated samples, in which the thiol groups were protected by the mixed disulfide, showed no change (samples 6 to 10). Optimum yields of the depolymerized product were expected to be obtained with 0.1*M* diethanoldisulfide and 0.004*M* ME.

Depolymerized Hp2-2 was therefore prepared in larger amounts by dissolving 55 mg of the protein in 5.5 ml of the borate-EDTA buffer (*p*H 8.7) and mixing with it an equal volume of the same buffer containing 0.2*M* diethanoldisulfide and 0.008*M* ME (added just before use). The mixture was incubated for 2 hours at 37°C in a petri dish (diameter, 8.5 cm) in which the solution was approximately 2 mm thick (to facilitate the oxidation of the ME during the incubation). Samples were taken at intervals and used later to confirm the progress of the reaction which was complete in about 90 minutes. After the incubation, dialyzable materials, including the diethanoldisulfide, were removed by passage through a Sephadex G25 column which had been previously washed with the diethanoldisulfide ME solution and then with distilled water. The product was freeze-dried and obtained in essentially 100 percent yield (51 mg).

Electrophoresis of solutions of the isolated product after the addition of progressively increasing amounts of hemoglobin established that the depolymerized Hp2-2 contained several components, all of which were able to bind hemoglobin. The major component (estimated as about 70 percent of the total protein) had a sedimentation coefficient (*10*) close to that of Hp1-1, the genetic variant of haptoglobin which occurs naturally in the unpolymerized form.

The reaction described by Eq. 4 appears, theoretically, to be applicable to any system in which disulfide-bond cleavage in proteins is to be studied, subject to the limitations of working in alkaline solutions. I have confirmed experimentally that it is applicable to several other proteins. Two Bence-Jones proteins, which I isolated from urine, and a defatted α_1-lipoprotein from serum (*11*) were successfully obtained as the corresponding mixed disulfides after treatment in 8*M* urea with 0.1*M* solutions of diethanoldisulfide or diaminodiethyldisulfide in the presence of 0.004*M* of their respective thiols in the borate-EDTA (*p*H 8.7) buffer.

Re-formation of the disulfide bonds cleaved during the preparation of the depolymerized haptoglobin was expected to be achieved by exposing the mixed disulfide product to catalytic amounts of mercaptoethanol (compare the observations of Kanarek *et al.*, *5*). Initial tests showed that considerable repolymerization could be obtained in the borate-EDTA buffer over a wide range of thiol concentrations (from 0.0002*M* to 0.006*M*) and that all the products were essentially equivalent. The products were not, however, completely repolymerized and gave electrophoresis patterns intermediate between those of depolymerized and untreated Hp2-2.

Reconsideration of the overall equilibrium in terms of the equation

$$n\text{Protein-S-S-R} \rightleftarrows \text{Protein-}\overset{\text{R-S}^-}{\text{S}}\text{-(S-Protein-S-)}_{n-2}\text{S-Protein} + (n/2)\ \text{R-S-S-R}$$

indicated that the equilibrium should be markedly dependent on the concentration of the reactant "monomer" (for example, to the cube of the concentration, if a trimer were to be formed). The repolymerization reaction was therefore repeated with the protein at a high concentration (0.5 mg of depolymerized Hp2-2 dissolved in 0.01 ml of borate-EDTA buffer containing 0.004*M* ME). A sample of "monomer" without ME and samples of native untreated Hp2-2 with and without ME were also prepared at the same concentration and in the same buffer. After approximately 20 hours at room temperature, the samples were diluted

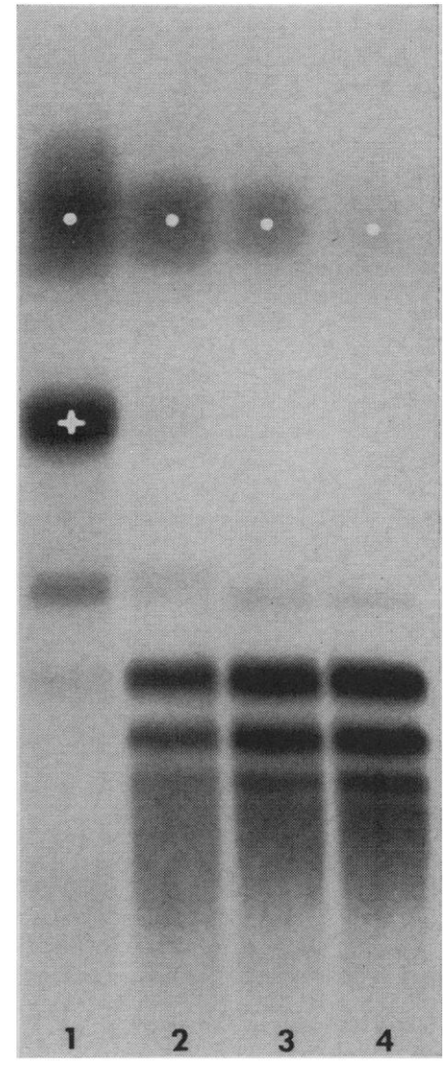

Fig. 2. Repolymerization of depolymerized haptoglobin. Benzidine-stained electrophoresis patterns after adding hemoglobin. (Sample 1) Depolymerized Hp2-2 in the form of its mixed disulfide with mercaptoethanol. Free hemoglobin is indicated by a dot, the major hemoglobin-haptoglobin complex by a cross. Lesser amounts of a complex migrating slightly faster than free hemoglobin and traces of complexes of much less mobility are visibile. (Sample 2) Repolymerized Hp2-2, prepared from sample 1 by exposure to a low concentration of mercaptoethanol. (Sample 3) Native Hp2-2, untreated except for exposure to the same low concentration of mercaptoethanol as sample 2. (Sample 4) Native Hp2-2, untreated. The repolymerized material is almost identical with the untreated Hp2-2 in its hemoglobin-binding and electrophoretic properties. The mixed disulfide of the depolymerized material is stable in the absence of added thiol.

with 0.1 ml of borate-EDTA buffer containing an excess of hemoglobin; their electrophoretic patterns are shown in Fig. 2. The re-formation of the disulfide bonds in the presence of 0.004*M* ME was almost perfect, as judged by the hemoglobin-binding and electrophoretic properties of the re-formed polymers; yet in the absence of added ME the mixed disulfide of the "monomer" was completely stable.

The use of the reaction shown in Eq. 3 to couple side chains to protein thiol groups was tested with hemoglobin and diaminodiethyldisulfide (cystamine). Hemoglobins A and A_2 have six cysteine residues per molecule: two of the six are readily accessible to reagents combining with thiols; the other four are inaccessible under mild conditions (*12*). Treatment of hemoglobin with cystamine [0.1*M* solution of the dihydrochloride in borate-EDTA (*p*H 8.7) buffer] at room temperature for 30 minutes was consequently expected to attach aminoethylsulfide side chains to the two accessible protein thiol groups. Prior treatment with iodoacetamide (0.01*M* in the same buffer) should prevent the reaction by alkylating the accessible thiol groups. Figure 3 shows the results of an experiment to test these expectations.

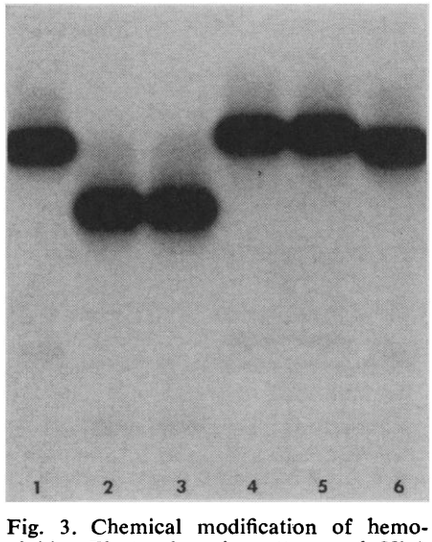

Fig. 3. Chemical modification of hemoglobin. Electrophoresis patterns of HbA and A_2 (the slower trace component) treated at room temperature as follows: (Sample 1) Borate-EDTA buffer alone; (sample 2) buffer plus diaminodiethyldisulfide (cystamine) for 30 minutes; (sample 3) cystamine for 30 minutes followed by iodoacetamide for 30 minutes; (sample 4) iodoacetamide for 30 minutes followed by cystamine for 30 minutes; (sample 5) iodoacetamide alone for 30 minutes; (sample 6) borate-EDTA buffer alone. Cystamine treatment attaches two positive side chains to both hemoglobins. Iodoacetamide prevents the coupling.

The iodoacetamide coupling slightly affects the electrophoretic mobilities of both hemoglobins A and A_2 (compare samples 5 and 6), presumably by affecting the dissociation of some of the ionizable amino acids in the proteins. Cystamine treatment has a considerable effect (samples 2 and 3 compared with 4 and 5), and the mobility changes are consistent with the addition of two positively charged groups per mole to both hemoglobins. (The chemically modified hemoglobin A migrates at a rate close to that of hemoglobin S, which has two fewer negatively charged residues than hemoglobin A.) Prior treatment with iodoacetamide prevents the cystamine coupling (no mobility differences between samples 4 and 5), and cystamine prevents iodoacetamide coupling (samples 2 and 3 have the same mobility). The results are completely consistent with expectations and show that 100-percent yields of an undenatured derivative of a thiol-containing protein can be obtained by mild treatment with a reagent disulfide under alkaline conditions.

These experiments also show that the determination of the electrophoretic mobilities of proteins after exposure to diaminodiethyldisulfide can be used to test for accessible thiol groups (preferably with diethanoldisulfide-treated material as the control). If the corresponding thiols are used in addition to the disulfides, the presence of accessible disulfide bridges in the proteins should also be detectable by still other mobility differences. Many variations in these procedures can be envisaged to investigate the relative accessibility of protein thiol groups or disulfide bridges under different conditions of *p*H, temperature, the presence of urea, and others.

The two groups of experiments which I have described here demonstrate the versatility and ease of control of the interchange reaction between a reagent disulfide and the disulfide bridges or thiol groups of proteins. Disulfide-bond cleavage, re-formation, and the chemical modification of proteins by the attachment of side chains can all be achieved by varying the reaction conditions (*13*).

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Electron Microscopic and Biochemical Characterization of Fraction I Protein

Abstract. *Electron micrographs of high resolution have been obtained from fraction I protein of Chinese cabbage leaves. The protein, which has ribulose-1,5-diphosphate carboxylase activity, appears to be a cube with edge of about 120 angstroms. Substructure can be seen in individual particles, consistent with a model having 24 subunits, the number prescribed by the available physical and chemical data.*

When crude extracts of tobacco leaves were examined in the analytical ultracentrifuge over a decade ago, a major soluble-protein fraction, termed the fraction I, showed a sedimentation coefficient of about 18*S* (*1*). Subsequent work showed that the fraction I protein is located predominantly, if not exclusively, in the chloroplast (*2*). There, it comprises by weight at least 50 percent of the soluble proteins (*2*, *3*). Fraction I protein has been reported to have the following biological activities: (i) protochlorophyll holochrome: the protein to which protochlorophyll is at-