Photodynamic Action of Bilirubin on Human Erythrocyte Membranes. Modification of Polypeptide Constituents†

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ABSTRACT: The photodynamic action of bilirubin on isolated human erythrocyte membranes (ghosts) has been studied. When incorporated into ghosts (pH 8.0, 10°) the bile pigment photosensitizes in blue light the peroxidation of unsaturated lipids, as evidenced by a positive color reaction with 2-thiobarbituric acid. Accompanying lipid peroxidation was the disappearance of most of the major membrane proteins (Coomassie Blue staining in sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and appearance of polypeptide photoproducts of greater size (mol wt >250,000). The association of membrane proteins (presumably by cross-linking) was insignificant when bilirubin–ghost suspensions were kept in the dark, or when ghosts were irradiated in the absence of bilirubin. Electrophoretic bands 1 and 2 (Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606) diminished rapidly during the photoreaction, whereas band 3 and the three sialoglycoproteins disappeared at a much slower rate. Dispersal of membrane constituents by treatment with sodium dodecyl sulfate prior to irradiation resulted in relatively little peroxidation and no noticeable formation of high molecular weight polypeptide complexes. The possibility that malonaldehyde, a product of lipid peroxidation, is involved in cross-linking during irradiation was studied by incubating ghosts with exogenous malonaldehyde. Although the reagent did cross-link membrane proteins (electrophoretic bands 1, 2, 2.1, 2.2, and 4.1 diminished most rapidly and high molecular weight bands appeared), the reaction could only be demonstrated with malonaldehyde concentrations several orders of magnitude greater than those detected in irradiation experiments. If malonaldehyde cross-linking occurs, it does not appear to be the predominant mechanism of polypeptide association during irradiation of bilirubin-containing ghosts.

The effect on protein structure of visible irradiation in the presence of oxygen and photosensitizing pigments (either synthetic or naturally occurring) has been the subject of numerous investigations. For the most part such studies have dealt with soluble proteins and enzymes (Weil and Seibles, 1955; Tu and McCormick, 1973; Mauk and Girotti, 1974), and relatively little has been done with membrane-bound polypeptides. Although photodynamic modification of membrane constituents (lipids and/or proteins) has long been suspected as the primary lesion during sensitized photodamage to cells (Blum and Hyman, 1939), the molecular mechanism of this modification is not well understood. The work reported herein focuses on this problem for the specific case of the bile pigment bilirubin in association with the human erythrocyte membrane.

The affinity of the erythrocyte for bilirubin is well known (Watson, 1962; Oski and Naiman, 1963), and it has been demonstrated (Saeki, 1932) that erythrocytes in vitro in the presence of bilirubin are readily hemolyzed when exposed to visible light. Recently Odell et al. (1972) have reexamined bilirubin-sensitized photohemolysis in conjunction with its possible occurrence during phototherapy for neonatal hyperbilirubinemia. These workers have observed that the action of bilirubin is truly photodynamic (i.e. requires molecular oxygen), and that the hemolytic process is initiated by membrane damage. In attempting to characterize this damage, they found that prior to hemolysis, there is a marked efflux of K^+ and inactivation of the Na^+,K^+-ATPase (the cation pump believed to play a central role in the establishment and maintenance of cellular morphology). Although ATPase activity was lost, it was not possible to distinguish between a direct effect on the enzyme itself and an indirect effect resulting from modification of surrounding membrane components. In the present study the photosensitizing action of bilirubin has been examined directly in the isolated erythrocyte membrane (ghost). A more comprehensive picture of membrane photodamage has been sought, with special attention being directed to the major membrane polypeptides and glycoproteins, i.e. those visualized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The results presented may provide new insight into how photosensitizing agents in general act on biological membranes.

Experimental Section

Chemicals. Bilirubin (reagent grade) was obtained from Schwarz/Mann. Analysis of this material by thin-layer chromatography (McDonagh and Assisi, 1971) revealed one major component (presumably bilirubin IX-α) and only traces of other components. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were Eastman products. Sodium dodecyl sulfate was obtained from Matheson Coleman and Bell (DX-2490) and from Fisher (S-329), and used without further purification. Dithiothreitol, 1,1,3,3-tetramethoxypropane, and 2-thiobarbituric acid were from Aldrich. All other chemicals were reagent grade and all solutions were prepared with glass-redistilled water.

Preparation of Erythrocyte Ghosts. Freshly drawn human blood in citrate–phosphate–dextrose solution was obtained from the Milwaukee Blood Center, and used within 2 weeks. Unsealed erythrocyte membranes (ghosts) were prepared according to the procedure of Dodge et al. (1963) with certain refinements described by Fairbanks et al.

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(1971). The lysing buffer was 6 m*M* sodium phosphate (pH 8.0), which was added at 4° to packed, washed erythrocytes in the volume ratio of 18:1. A total of three washings with 18 vol of 6P8[1] (centrifugation at 22,000*g* for 30 min) gave ghosts that were essentially hemoglobin free. Membrane protein was determined by the method of Lowry et al. (1951) after solubilization of ghost samples in 0.2% sodium dodecyl sulfate. Bovine serum albumin served as the standard protein. Stock suspensions usually contained 3–5 mg of ghost protein/ml. Ghosts were generally used within 1 week after preparation.

Bilirubin-Charged Ghosts. Stock solutions of bilirubin (0.5–1.0 mg/ml) were prepared in dim light by dissolving the bile pigment with 2 molar equiv of NaOH, and diluting quickly with 6P8. These solutions were used within 1 hr. The following steps were carried out at 4° in minimally illuminated surroundings. An aliquot of bilirubin (either 0.1 or 1.0 equiv on the basis of GP[1] weight) was added to a sample of ghosts and the mixture diluted with 6P8 to the desired concentration of GP. After incubation for 15 min with occasional mixing, the ghosts were pelleted (35,000*g* for 20 min), washed at least once with 6P8, and resuspended in this buffer to a final concentration between 0.5 and 2 mg of GP/ml. Spectral determination of bilirubin in the supernatants revealed that approximately 90% of the bile pigment, when added originally as 0.1 GP equiv, is retained by the membranes.

Irradiation Procedure. Photoreactions were carried out in a thermostated beaker (35 mm diameter) at 10 ± 1°. The light source was a 250-W Duro Test lamp (Model R40) positioned approximately 15 cm above the sample. Lamp heat was dissipated continuously with a fan. A blue filter (Corning, CS No. 5-57) was placed between the lamp and sample. This restricted the incident light to wavelengths near λ_{max} of bilirubin. Irradiance near the sample surface (~100 J m^{-2} sec^{-1} for all experiments) was measured with a YSI Radiometer, Model 65A. Each bilirubin-ghost suspension (2–3 ml) was transferred to the reaction vessel, convered with a sheet of clear polyethylene, and purged with water-saturated O_2 for 15 min in the dark. During irradiation, the suspension was stirred magnetically while a stream of O_2 was passed over its surface. For experiments conducted in the absence of O_2, argon (scrubbed through alkaline pyrogallol to remove traces of O_2) was bubbled through bilirubin-charged ghosts for 1 hr, and then diverted over the suspension during illumination.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Prior to electrophoresis ghost samples (with or without bilirubin present) were solubilized as follows (Fairbanks et al., 1971). To one volume of ghost suspension was added 0.67 vol of a solution containing 2.5% sodium dodecyl sulfate, 25 m*M* Tris-HCl (pH 8.0), 2.5 m*M* EDTA, 100 m*M* dithiothreitol, 25% sucrose, and 0.005% Bromophenol Blue (tracking dye). The dissolved membranes were then heated at 37° for 30 min to accelerate reduction of disulfide bonds. Aliquots of these solutions (15–30 μg of GP for analysis of polypeptides and 60–100 μg for the glycoproteins) were subjected to polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate. The procedure followed was essentially that of Fairbanks et al. (1971). Gels (0.75 cm diameter × 10 cm long) were 5.6% in acrylamide and 1% in sodium dodecyl sulfate. After polymerization these were overlaid with electrophoresis buffer (consisting of 40 m*M* Tris-HCl (pH 7.4), 20 m*M* sodium acetate, 2 m*M* EDTA, and 1% sodium dodecyl sulfate) and allowed to stand overnight before electrophoresis. Samples were allowed to migrate into the gels at 1 mA/gel for 15 min; the current was then raised to 5 mA/gel for the remainder of the run, i.e. until tracking dye had moved ~9 cm. After removal from their tubes, the gels were fixed and stained (either with Coomassie Blue for simple polypeptides or with PAS[1] for glycoproteins) and then destained and stored as described previously (Fairbanks et al., 1971). Gel densitometry was carried out with a Gilford linear transport accessory (Model 2410-S) used in conjunction with a Gilford spectrophotometer (Model 240) and recorder (Model 6040). Coomassie Blue bands and PAS bands were scanned at 550 and 560 nm, respectively.

Lipid Peroxidation. End products of peroxidation of polyunsaturated fatty acids react with 2-thiobarbituric acid to produce a pink complex (Dahle et al., 1962). This reaction is widely used as an index of lipid peroxidation in membrane systems. The principal reactant with thiobarbituric acid is recognized as malonaldehyde (Kwon and Olcott, 1966). In the present study the thiobarbituric acid assay was modeled after that of Placer et al. (1966). To each sample of irradiated ghosts (0.5–1.0 mg of GP) were added 2.0 ml of pyridine–butanol (3:1, v/v) for solubilization, 1.0 ml of thiobarbituric acid reagent (0.5% thiobarbituric acid in 2.5% $HClO_4$), and H_2O to 4.0 ml final volume. The samples were heated in a boiling water bath for 10 min and cooled 20 min and their absorbancies at λ_{max} (539 nm) recorded. Membrane malonaldehyde concentrations were calculated using $\epsilon_{539} = 166$ mM^{-1} cm^{-1}. This extinction coefficient was determined by treating thiobarbituric acid with standardized malonaldehyde, which was generated from malonaldehyde bisdimethyl acetal (Kwon and Watts, 1963). The contribution of membrane-bound bilirubin to A_{539} was found to be insignificant.

Malonaldehyde Reactions. The following procedure was carried out to ascertain whether malonaldehyde would cross-link ghost proteins. Stock solutions of malonaldehyde (~12 m*M*) were prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane, e.g. incubation in 5 m*M* HCl overnight at 25°. Malonaldehyde concentrations were checked spectrophotometrically at 267 nm (pH 9), using an extinction coefficient of 31 mM^{-1} cm^{-1}. Prior to addition to ghosts, malonaldehyde solutions were neutralized in sodium phosphate buffer (40 m*M*). In a typical experiment a malonaldehyde-ghost suspension (containing 1 mg of GP/ml; 10 m*M* malonaldehyde; and 33 m*M* sodium phosphate, pH 6.9) was stirred in a thermostated (25°) beaker; at various intervals samples were centrifuged, washed with and resuspended in 6P8, and then analyzed by sodium dodecyl sulfate gel electrophoresis as described above. A control lacking malonaldehyde, but containing the appropriate concentrations of methanol (to account for methanol generated from hydrolysis of the acetal) and phosphate buffer, was incubated and analyzed simultaneously.

Results

Effect of Irradiation on Membrane Proteins. The disposition of the major ghost polypeptides after various periods of irradiation in the presence of bilirubin was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The results of a typical experiment are shown in Figure 1. It can be seen (gels A to D) that there is a progressive loss of

[1] Abbreviations used are: 6P8, 6 m*M* sodium phosphate buffer (pH 8.0); GP, ghost protein; PAS, periodate–Schiff reagent.

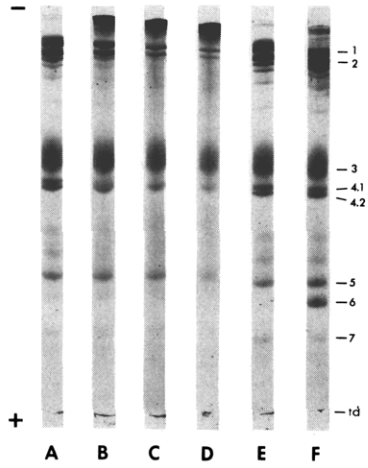

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of membrane proteins after irradiation of ghosts in the presence of bilirubin. After addition of bilirubin (0.95 mM), ghosts (0.5 mg of GP/ml in 6P8) were washed twice with 6P8, resuspended in buffer to the original volume, and irradiated for the following periods (minutes): 0 (A); 60 (B); 120 (C); 240 (D). Controls: E is a sample incubated for 240 min in the dark; F represents ghosts irradiated 240 min in the absence of bilirubin. Sample load: 15 μg of GP/gel. Position of tracking dye (TD) was marked with drawing ink. Bands are numbered according to Steck (1972).

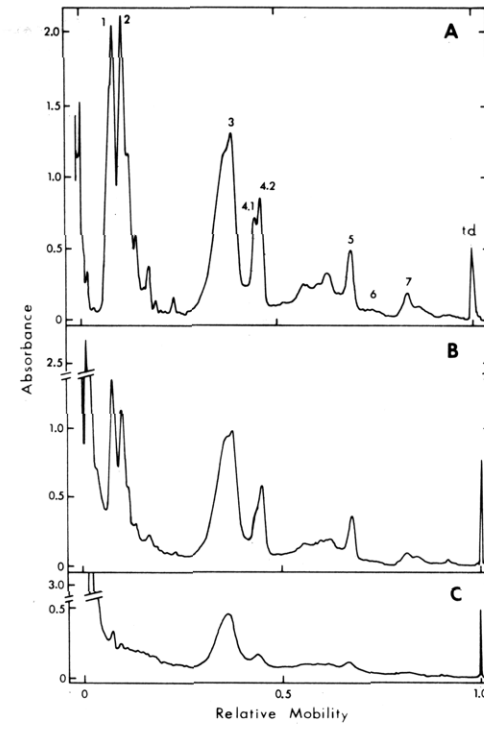

FIGURE 2: Electrophorograms of Coomassie Blue staining polypeptides after irradiation (0 min, A; 15 min, B; 180 min, C) of bilirubin-charged ghosts. After mixing with bile pigment (3.4 mM), ghosts (2 mg of GP/ml) were washed and irradiated as described in Figure 1; GP per gel, 24 μg. Gels were scanned at 550 nm. Mobility is relative to that of tracking dye (TD).

all protein bands[2] (Coomassie Blue staining) and appearance of high molecular weight (>250,000) material near the origin of the gels. In control experiments (ghosts + bilirubin prepared in the same manner and kept in the dark, gel E; and ghosts irradiated in the absence of bilirubin, gel F), relatively little, if any, effect is observed. Also when ghosts + bilirubin were irradiated in an argon atmosphere (not shown) the apparent cross-linking of membrane polypeptides was sharply reduced.[3] Qualitatively the same results seen in Figure 1 were obtained when ghosts were originally treated with one-tenth the concentration of bilirubin. Some protein bands were found consistently to diminish to a greater extent than others. This is illustrated in Figure 2, which shows densitometric tracings of gels taken from a separate experiment. Integration of peak areas reveals that after 3 hr of irradiation, less than 2% remains of polypeptides 1 + 2, collectively called "spectrin" (Marchesi and Steers, 1968), whereas 30% of component 3 is left. It is noteworthy that even before the onset of illumination, at least one faint band of high molecular weight polypeptide(s) was usually observed immediately below the origin (Figure 2A). This band cannot be ascribed to a dark effect of bilirubin, since it was also seen with bilirubin-free ghosts. Although found in electrophorograms prepared by others (Fairbanks et al., 1971), it has received little attention. After 15 min of illumination in the presence of bilirubin, the amount of material at this position had already increased markedly (Figure 2B); however, no new bands of intermediate molecular weight (i.e. between bands 1 and 7) were evident. Photoreactions of shorter duration have not been studied. In an attempt to separate polypeptide photoproducts, preliminary electrophoretic runs on composite gels of 0.4% agarose–3.5% polyacrylamide (Steck, 1972) have been carried out. In one experiment (not shown) bilirubin-charged ghosts were analyzed after irradiating for 90 min; three new bands (estimated mol wt 390,000, 370,000, and 330,000) were observed. However, a large amount of material barely entered the gels and still remained unresolved. Since polypeptides 1 and 2, at 240,000 and 215,000 g/mol, respectively (Steck, 1972), disappeared rapidly, it is conceivable that the above three bands could have arisen from a cross-linking reaction involving 1 or 2 and smaller components.

Certain additional observations on the Coomassie Blue reactive photoproducts have been made. They do not appear to consist predominantly of polypeptides linked by disulfide bonds, since reduction with dithiothreitol (40 mM) was carried out prior to electrophoresis. That any cross-linking at all could have been attributable to S–S bonds was tested in one experiment by omitting the preincubation with dithiothreitol. The gels in this case were not markedly different from those prepared with reduced samples, suggesting that disulfide cross-linking does not occur to any appreciable extent.

When irradiated ghosts were washed repeatedly with cold 6P8 before analysis, their electrophoretic patterns were indistinguishable from those of unwashed ghosts (Figures 1 and 2), i.e. there was no noticeable elution of high molecular weight material. Although not conclusive, this evidence suggests that the polarity of this material was not signifi-

[2] The behavior of band 6 in experiments of this type is deserving of comment. Even before irradiation only a trace of this component is seen. It is now clear (A. W. Girotti, manuscript in preparation) that band 6, the monomer of glyceraldehyde-3-phosphate dehydrogenase (Tanner and Gray, 1971; Kant and Steck, 1973) is eluted selectively and virtually completely from ghosts by bilirubin concentrations of 1 mM or greater (compare gel F with gel E in Figure 1). This is not an ionic strength effect such as that observed with isotonic saline (Kant and Steck, 1973), but may be attributed to weakened binding of the enzyme as the membrane interacts with bilirubin.

[3] Invariably a small amount of high molecular weight material was observed in this case; this result is attributed to residual O_2 which remained in the membranes despite prolonged purging with Ar.

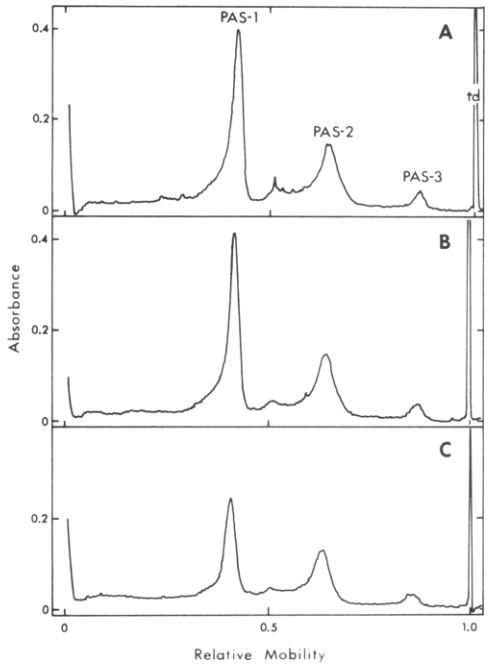

FIGURE 3: Electrophorograms of the sialoglycoproteins after irradiation (0 min, A; 15 min, B; 180 min, C) of bilirubin-charged ghosts (prepared as described in Figure 2); GP per gel, 88 μg. Gels were scanned at 560 nm. Bands are numbered according to Fairbanks et al. (1971).

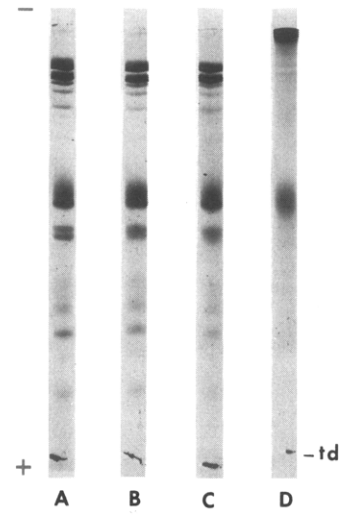

FIGURE 4: Polypeptide electrophoretic patterns of bilirubin-containing ghosts solubilized in 1% sodium dodecyl sulfate prior to irradiation (see Figure 1 for experimental details). Reaction periods (minutes): 0 (A); 60 (B); and 240 (C). In gel D irradiation (240 min) was carried out in the absence of sodium dodecyl sulfate; sample load, 15 μg of GP/gel.

cantly greater than that of the polypeptides from which it was derived.

The effect of irradiation on the membrane glycoproteins has been examined. As can be seen in the electrophoretic patterns in Figure 3, there is no detectable change in any of the three major glycoproteins (PAS-1, PAS-2, and PAS-3) after irradiating for 15 min. After 3 hr approximately 50% of PAS-1 was left, whereas PAS-2 and PAS-3 remained practically unchanged. It is apparent from these results that the glycoproteins as a whole are considerably less sensitive to the photodynamic effects of bilirubin than are, e.g., polypeptides 1 and 2. In this sense they resemble component 3 (see above). Although not evident in Figure 3C, the partial loss of PAS-1 was accompanied by the appearance of a diffuse carbohydrate-containing band near the origin, indicating that at least some of the PAS-1 had become incorporated into the cross-linked photoproduct.

It was of interest to determine whether membrane solubilization before irradiation would have any effect on the formation of polypeptide aggregates. As can be seen in Figure 4, prior treatment of bilirubin-containing ghosts with 1% sodium dodecyl sulfate resulted in relatively little change in the normal polypeptide electrophoretic pattern (compare gels A, B, and C), whereas without detergent (gel D) there was again a loss of all bands (except band 3) and appearance of very high molecular weight material. It is evident from these results that the proximity of various polypeptides to one another (and also to bilirubin) in the "native" ghost membrane is necessary for efficient photoinduced cross-linking to occur.

Lipid Peroxidation. During irradiation of bilirubin-charged ghosts under conditions shown to produce cross-linked polypeptides, the thiobarbituric acid reaction indicated that malonaldehyde was generated (Figure 5). Although the yield of malonaldehyde appears small (~5 nmol/mg of GP after 2 hr) when compared with the average level of the aldehyde detected before irradiation (legend, Figure 5), this evidence nevertheless demonstrates that peroxidation of unsaturated lipids took place in the membranes. The plateau in the plot after 2 hr does not appear to be due to depletion of peroxidizable lipids, but rather to photodestruction of bilirubin itself (McDonagh, 1971), since additional bilirubin after 4 hr resulted in a second step in malonaldehyde formation. Control experiments carried out in the absence of bilirubin or O_2 (Figure 5) indicated that relatively little or no malonaldehyde was produced under these conditions. Furthermore, when solubilization of ghosts in 1% sodium dodecyl sulfate preceded irradiation, the output of malonaldehyde decreased by approximately 80%. The fact that these observations parallel those made electrophoretically on ghost proteins (Figures 1, 2, and 4) suggests that lipid peroxidation and polypeptide cross-linking are not independent processes, but are related in a cause and effect manner. From the data obtained here, it is not possible to distinguish between the time courses of these processes. It has been reported, however, that in general during oxidative damage in biomembrane systems, lipid peroxidation precedes and is ultimately responsible for a variety of changes in membrane proteins and enzymes, e.g. cross-linking, polypeptide chain scission, and modification of side-chain groups (Tappel, 1973).

Cross-Linking with Malonaldehyde. Bifunctional aldehydes such as malonaldehyde are known to be effective cross-linking agents of soluble proteins such as serum albumin (Kwon and Brown, 1965) and ribonuclease (Chio and Tappel, 1969). Tappel (1973) has suggested that malonaldehyde, generated as a secondary product of lipid peroxidation in biomembranes, might act similarly on membrane proteins. However, this has not been positively demonstrated for malonaldehyde as it has (in vitro) for other aldehydes such as formaldehyde and glutaraldehyde (Steck, 1972; Capaldi, 1973). As an aid in understanding whether malonaldehyde might play any significant role in cross-linking when bilirubin-containing ghosts are irradiated, experiments were carried out in which ghosts were incubated with exogenous malonaldehyde. As can be seen in the sodium dodecyl sulfate electrophoretic patterns in Figures 6, malonal-

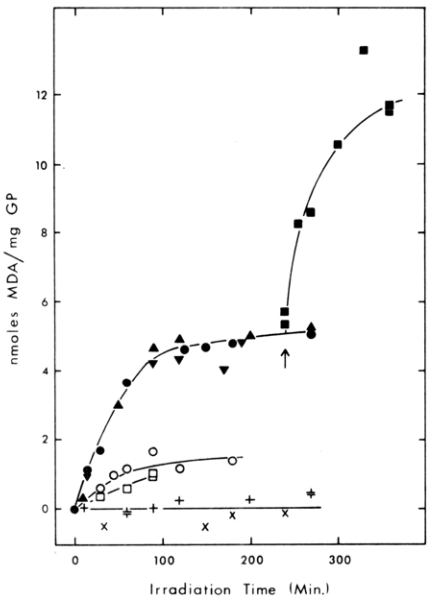

FIGURE 5: Malonaldehyde (MDA) formation upon irradiation of ghosts (1.5 mg of GP/ml) in the presence of bilirubin (0.26 mM). Data from four separate experiments are shown (●, ■, ▲, ▼). Values are corrected for malonaldehyde present at zero time (varied from 3.6 to 10.9 nmol/mg of GP in different ghost preparations). After 4 hr in one experiment (arrow), additional bilirubin (0.26 mM) was added. Also shown are data from experiments conducted in the absence of bile pigment (+,×), in the absence of oxygen (□), and in the presence of 1% sodium dodecyl sulfate (○).

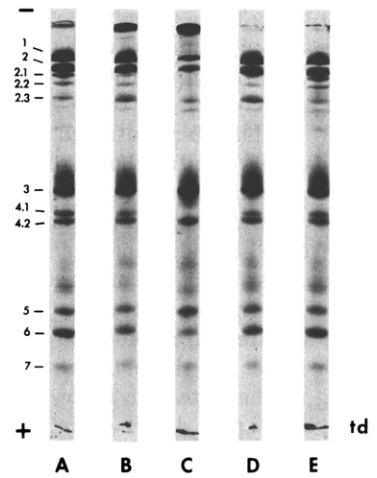

FIGURE 6: Electrophorograms obtained after incubation (25°) of ghosts (1.1 mg of GP/ml) in 9.5 mM malonaldehyde for 30 min (A); 5 hr (B); and 23 hr (C). Gel D shows pattern after 5 hr in the absence of malonaldehyde; gel E after 5 hr in 9.1 mM malonaldehyde + 1% sodium dodecyl sulfate; sample load, 17 μg of GP/gel.

dehyde, under the conditions used, was capable of cross-linking ghost proteins. The amount of cross-linked material (which is seen close to the origins of gels A, B, and C) increased gradually throughout the incubation period of 23 hr, and various protein bands either disappeared or became diminished. Fine resolution of reaction products was not possible in the gels used; however, a slight separation of components is evident in gel A. The reaction appeared to occur more slowly than that observed previously with other aldehydes (Steck, 1972). It may be for this reason that Steck (1972) failed to detect cross-linking of ghost proteins with malonaldehyde. In a control experiment without malonaldehyde, no higher molecular weight polypeptides appeared after 5 hr (Figure 6D). Also, when ghosts were solubilized in sodium dodecyl sulfate before the reaction, cross-linking was abolished (Figure 6E). This observation resembles the one made in irradiation experiments (Figure 4), and is similar to that reported by Steck (1972). As seen in Figure 6 certain proteins disappeared more rapidly than others during the malonaldehyde reaction. The situation is broadly similar to that observed during irradiation (Figures 1 and 2). For example, there was a sizable loss of components 1 and 2 over 23 hr, but little real loss of component 3. The slightly greater mobility of the latter may have been caused by trace protease activity (Fairbanks et al., 1971) during prolonged incubation at 25°. The bulk of this activity appears to have been inhibited by malonaldehyde, since relatively extensive proteolysis was evident after 23 hr in the absence of the aldehyde. Curiously component 5, which is believed to be associated in some manner with 1 and 2 in the membrane (Steck et al., 1971), did not diminish along with the latter two, an observation also made with glutaraldehyde (Steck, 1972). Bands 2.1, 2.2, and 4.1 were missing after overnight incubation with malonaldehyde, and band 6 appeared reduced. On the other hand this treatment had no noticeable effect on the three glycoproteins (not shown). The latter finding is consistent with that obtained with other cross-linking agents (Steck, 1972; Capaldi, 1973), and agrees qualitatively with the irradiation results on the glycoproteins (Figure 3).

The appearance of malonaldehyde cross-linked polypeptides was not only slow, but required concentrations of aldehyde considerably higher than those detected following irradiation of bilirubin-charged ghosts. In the photoreactions shown in Figure 5, for example, approximately 10 μM malonaldehyde was generated after 2 hr. When bilirubin-free ghosts were incubated with comparable concentrations of malonaldehyde, no cross-linking such as that in Figure 6 was observed. In fact, a reaction was barely noticeable when 1 mM malonaldehyde was used. This evidence, although not conclusive in itself, is suggestive that cross-linking through malonaldehyde (if it occurs at all) is not the predominant mechanism by which polypeptide complexes are formed during irradiation. In support of this conclusion is the fact that invariably appreciable malonaldehyde was detected in ghosts prior to irradiation (cf. legend, Figure 5), and yet the amount of high molecular weight material was relatively insignificant. Possible alternative cross-linking schemes are discussed below.

Discussion

Evidence has been presented that bilirubin is a potent sensitizer of photodynamic damage in the red cell ghost. This damage has been characterized as lipid peroxidation and polypeptide cross-linking. Whether such reactions also take place in the intact cell is currently being investigated in this laboratory. Recent work (A. W. Girotti, unpublished data) has revealed that several enzymes (Mg^{2+}-ATPase, Na^+,K^+-ATPase, acetylcholinesterase, and glyceraldehyde-3-phosphate dehydrogenase) in the isolated membrane are inactivated by irradiation in the presence of bilirubin. Although the nature of any of these inactivations is not known, it is conceivable that intra- or intermolecular cross-linking is involved. Photoinduced cross-linking such as that described here has apparently not been reported previously with any type of dye–membrane system. The effect is not due to some unique property of bilirubin. This follows from

the preliminary finding that polypeptide complexes are also formed when ghosts are irradiated in the presence of Rose Bengal (A. W. Girotti, unpublished data). As already pointed out, it is unlikely that the complexes observed electrophoretically in the bilirubin-sensitized reaction (Figures 1 and 2) are joined by disulfide bonds, because reduction was carried out before analysis. The effect of irradiation on membrane proteins could be mimicked by incubating ghosts with malonaldehyde, a secondary product of lipid peroxidation and a known cross-linking agent (Figure 6). It was concluded, however, that the concentrations of this compound necessary to see an effect are much too high to have any significance with respect to the photoreaction. As alternative cross-linking mechanisms, the following could be considered (Roubal and Tappel, 1966; Zirlin and Karel, 1969): (i) interaction of membrane polypeptides (P) with lipid (L) peroxy radicals to give structures such as $-P-O_2-L-O_2-P'-$; or (ii) chain polymerization-type reactions of polypeptide radicals (possibly generated by free-radical intermediates of lipid peroxidation) to give structures of the type $\cdot P''-P'-P$. No evidence supporting free-radical schemes such as these has been obtained as yet. However, the effect on the photoreaction of free-radical inhibitors, e.g. α-tocopherol and butylated hydroxytoluene (Leibowitz and Johnson, 1971; Anderson and Krinsky, 1973), is currently being studied in this laboratory.

The possibility that cross-linking is accompanied by other modifications, e.g. photooxidation of susceptible amino acid residues (His, Cys, Met, Tyr, Trp), has not yet been examined. However, it has been shown (Schothorst and van Steveninck, 1972; Goldstein and Harber, 1972) that such residues can be destroyed by irradiating porphyrin-containing ghosts. There is no electrophoretic evidence of formation of lower molecular weight peptides during the photoreaction with bilirubin (Figure 2). One can conclude, therefore, that polypeptide chain scission, which is possible in systems undergoing free-radical reactions (Tappel, 1973), does not occur in this case.

Polypeptides 1 and 2, which occupy the inner face of the membrane and account for about 25% of the total protein (Fairbanks et al., 1971), diminished most rapidly during irradiation (Figure 2). It is likely, therefore, that the bulk of the cross-linked material early in the reaction consisted of homo- or heterocomplexes of these components. A similar rapid disappearance of 1 and 2 has been noticed after treatment of ghosts with cross-linking reagents (Steck, 1972; Wang and Richards, 1974). By contrast the transmembrane proteins, i.e. band 3 and the three sialoglycoproteins, changed relatively little during the photoreaction (Figures 3 and 4D). The sluggish reactivity of band 3 is to be compared with previous observations on this component. It has been shown (Steck, 1972) that polypeptide 3 cross-links relatively slowly with reagents such as formaldehyde and glutaraldehyde. On the other hand, treatment of ghosts with Cu^{2+}-*o*-phenanthroline, which catalyzes the oxidation of sulfhydryl groups to disulfides, causes a rapid conversion of component 3 to homopolymers, primarily dimers (Steck, 1972; Wang and Richards, 1974). Such products have not been observed in the present case. The behavior of the glycoproteins is equally interesting and is consistent with findings that these components are essentially unreactive toward a variety of cross-linking agents (Steck, 1972; Capaldi, 1973; Wang and Richards, 1974), including malonaldehyde (see Results). The present results support previous views (Capaldi, 1973) that the glycoproteins as a whole tend to migrate freely in the lipid phase of the membrane and make little contact with other proteins.

The photohemolytic action of various lipophilic pigments on isolated human erythrocytes has been studied by numerous investigators. The pigments tested have been either synthetic dyes such as Rose Bengal (Blum and Morgan, 1939; Blum and Hyman, 1939), or natural metabolites such as protoporphyrin IX (Hsu et al., 1971; Goldstein and Harber, 1972; Lamola et al., 1973) or bilirubin (Odell et al., 1972). Certain general observations have been made. (i) A prelytic lesion occurs somewhere within the plasma membrane, where the sensitizing agent is usually bound. (ii) This reaction is photodynamic, i.e. it requires oxygen and visible light of wavelength near λ_{max} of the pigment. (iii) Lipid peroxidation usually takes place; this may play a key role in cellular breakdown (Hsu et al., 1971; Lamola et al., 1973). The consequence of peroxidation could be disruption of the lipid bilayer per se; on the other hand, lipid free-radical intermediates could trigger the denaturation of important membrane proteins and enzymes (Tappel, 1973). Although information pertaining to photohemolysis has accumulated rapidly in recent years, the overall mechanism of the process at the molecular level is still far from clear. The present findings, particularly those relating to membrane proteins, may provide new insight into this problem.

Interest in photohemolysis sensitized by natural pigments has been stimulated by possible clinical ramifications. In the case of protoporphyrin, for example, the in vitro process has been looked upon as a model of cellular photodamage that often occurs in the disorder erythropoietic protoporphyria when surface tissues are exposed to light. The bilirubin-sensitized reaction has attracted considerable attention lately because of the possible significance of hemolysis during phototherapy for neonatal hyperbilirubinemia (Kopelman et al., 1972). The objective of phototherapy in this disorder is to reduce bilirubin in the serum by effecting its photooxidation in the skin and surface circulation. Photooxidation of bilirubin in vitro is known to occur via a self-sensitized mechanism involving singlet molecular oxygen (McDonagh, 1971). This type of reaction in vivo could conceivably result in damaging side effects, not only to erythrocytes, but to other cells that normally make contact with the bile pigment. Whether such effects constitute potential hazards of phototherapy has been a topic of vigorous debate in recent years (Behrman et al., 1974; McDonagh, 1974). The present work was restricted to erythrocyte membranes under conditions far from physiological. Nevertheless, these results, which clearly illustrate the diverse membrane photodamage initiated by bilirubin, could prove relevant to the above issue.

Acknowledgment

The author is grateful to Mrs. Barbara Makovec for her excellent technical assistance.

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