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Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.
Prestegard, J. H., & Fellmuth, B. (1974) *Biochemistry 13*, 1122–1126.
Rand, R. P., Reese, T. S., & Miller, R. G. (1981) *Nature (London) 293*, 237–238.
Schmidt, C. F., Lichtenberg, D., & Thompson, T. E. (1981) *Biochemistry 20*, 4792–4797.
Shaw, J. M., Hutton, W. C., Lentz, B. R., & Thompson, T. E. (1977) *Biochemistry 16*, 4156–4163.
Shick, P. K. (1978) *J. Lab. Clin. Med. 91*, 802–810.
Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., & Papahadjopoulos, D. (1980) *Biochim. Biophys. Acta 601*, 559–571.
Valentine, R. C., Shapiro, B. M., & Stadtman, E. R. (1968) *Biochemistry 7*, 2143–2151.
Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta 515*, 163–205.

Glycoprotein Topology on Intact Human Red Blood Cells Reevaluated by Cross-Linking following Amino Group Supplementation†

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ABSTRACT: Protein–protein interactions were studied at 0–4 °C with amino group specific cross-linkers on intact human erythrocytes after introducing free alkylamino groups into glycoproteins to overcome the scarcity of accessible amino groups. Amino group supplementation is based on the formation of aldehydes by either enzymatic or chemical oxidation. Subsequently, an imine is formed between aldehyde groups and 2-(4-aminophenyl)[1-^{14}C]ethylamine (arylalkyldiamine) in the presence of sodium cyanoborohydride ($NaCNBH_3$). The arylamino group of arylalkyldiamine forms imines at least 280 times more rapidly than the alkylamino group. This property leaves the majority of alkylamino groups free for subsequent cross-linking with bifunctional reagents. Amino group supplementation enhances the cross-linking probability of glycophorins in glycophorin-containing vesicles. When applied to intact human erythrocytes of any age, in conjunction with the cross-linker disuccinimidyl 3,3′-dithiobis(propionate), the glycoproteins, band 3 and glycophorins, did not undergo substantial cross-linking (less than 2% of the total label cross-linked). However, substantial cross-linking (20% of the total label cross-linked) of either glycoprotein was detected on spectrin-free vesicles [Lutz, H. U., Liu, S. C., & Palek, J. (1977) *J. Cell Biol. 73*, 548–560] that are devoid of cytoskeletal restraints. The inability to cross-link these proteins on intact cells is not due to a lack of accessible amino groups on the surface of these glycoproteins, because amino group supplementation enhanced their ability to form cross-links on intact cells and on spectrin-free vesicles by the same factor (2–3-fold). This and various controls suggest a monomeric arrangement of the exoplasmic portions of band 3 on *intact* erythrocytes.

Chemical cross-linking of memrane proteins has been studied extensively on isolated erythrocyte membranes (Steck, 1972; Ji, 1973; Wang & Richards, 1974; Staros et al., 1974; Lutz et al., 1977a). These investigations have generated a considerable knowledge of possible protein–protein interactions within the membrane, among them the existence of band 3 dimers (Wang & Richards, 1974; Nigg & Cherry, 1979) but an absence of interactions between glycophorins (Steck, 1972; Capaldi, 1973). However, only a limited number of experiments were carried out on intact cells (Wang & Richards, 1975). These studies revealed essentially the same results for intact cells and unsealed ghosts but were hampered by the fact that permeable reagents caused extensive cross-linking of cytoplasmic proteins and spectrin. Thus, concomitant cross-linking of band 3 could have been due to cytoplasmic cross-links or could occur passively as a consequence of cross-links within the cytoskeletal framework. Staros et al. (1981) recently applied an impermeable bifunctional cross-linking reagent to intact cells and could demonstrate band 3 cross-linking without simultaneously cross-linking cytoskeletal elements (Staros et al., 1981). This was taken as evidence for band 3 dimers on native membranes, although the conditions used to detect it (30 min at room temperature) clearly allowed long-range protein mobility to occur. Hence, these data cannot exclude the possibility that monomeric band 3 protein formed cross-linkable dimers in the course of diffusion-controlled collisions. Collisions of monomeric band 3 could occur at this elevated temperature in media that do not allow the cells to maintain their natural ATP content. Extensive ATP depletion is known to facilitate cross-linking of membrane proteins in intact cells as measured by endogenous SS-bridge formation at pH values close to the isoelectric point of the proteins involved (Liu et al., 1977). The glycoprotein topology of intact erythrocytes is further complicated by the fact that negligible extents of glycophorin cross-links were detectable (Ji, 1979). This was tentatively ascribed to an inaccessibility of endogenous amino groups (Marchesi & Furthmayr, 1976).

For these reasons we have developed a method to overcome the scarcity of accessible, endogenous amino groups by supplementing surface glycoproteins on intact cells with covalently

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attached free alkylamino groups. This method, applied in conjunction with the reactive bifunctional cross-linker DTSP[1] (Lomant & Fairbanks, 1976), allowed us to probe the glycoprotein topology at 0–4 °C, where long-range protein mobility is practically inexistent (Peters, 1981). In this report we describe the technique of amino group supplementation and demonstrate that introduction of additional amino groups in fact enhances the cross-linking probability of glycophorins in glycophorin-containing vesicles. However, when applied to intact cells, a very small cross-linkability for either band 3 or glycophorin was detected, irrespective of amino group supplementation. This essentially negative result is supported by the occurrence of extensive cross-links for both glycoproteins on spectrin-free vesicles that lack the cytoskeleton but are otherwise comparable to intact erythrocytes (Lutz et al., 1977b).

Materials and Methods

Chemicals and Enzymes. [U-^{14}C]Aniline hydrogen sulfate (98.8 mCi/mmol) and NCS-tissue solubilizer were purchased from the Radiochemical Centre, Ltd., Amersham, England. 2-(4-Aminophenyl)[1-^{14}C]ethylamine dihydrochloride (58 mCi/mmol) was synthesized upon request at the Radiochemical Centre, Ltd., Amersham. 2-(4-Aminophenyl)[1-^{14}C]-ethylamine dihydrochloride was prepared from potassium [^{14}C]cyanide and benzyl chloride via benzyl [^{14}C]cyanide and *p*-nitrobenzyl [^{14}C]cyanide. The latter was reduced with hydrogen and Adams catalyst in acetic anhydride, and the crude product was obtained by refluxing with hydrochloric acid. It was purified by paper chromatography. Phenyl[1-^{14}C]ethylamine hydrochloride (48.25 mCi/mmol) was obtained from New England Nuclear. Sodium metaperiodate was purchased from Sigma (St. Louis, MO); DTSP was from Pierce Chemical Co. (No. 22585). Sodium cyanoborohydride and 2-(4-aminophenyl)ethylamine were obtained from Aldrich-Europe (Beerse, Belgium). Galactose oxidase (EC 1.1.3.9), Type V, from *Dactylium dendroides*, was purchased from Sigma or from Millipore-Worthington Corp., Freehold, NJ. Lyophilized galactose oxidase was transferred from container to tube under an atmosphere of argon to prevent loss of activity. Neuraminidase (EC 3.2.1.18) from *Vibrio comma* (cholerae) was from Behring Werke AG, Marburg, West Germany.

Isolation of Erythrocytes, of Erythrocyte Subpopulations, of Spectrin-Free Vesicles, and of Glycophorin-Containing Vesicles. Human blood, O Rh$^+$, obtained from the Swiss Red Cross Zurich, had been collected in citrate–phosphate–dextrose (CPD). Red blood cells were spun down and washed 3 times in phosphate-buffered saline (150 mM NaCl–5 mM $NaKHPO_4$, pH 7.4).

Separation of erythrocytes according to their density was carried out by centrifugation on self-forming gradients of Percoll (Pharmacia, Uppsala) as described elsewhere (Lutz & Fehr, 1979). Young (light) and senescent (dense) erythrocytes were assayed for their creatine contents as a cell age parameter (Fehr & Knob, 1979).

Spectrin-free vesicles were obtained by ATP depletion of erythrocytes in a glucose-free medium for 40 h at 37 °C according to Lutz et al. (1977b) under pH-maintaining conditions described elsewhere (Müller et al., 1981).

Glycophorin-containing vesicles were obtained with coextracted lipids from these membranes as described elsewhere (Lutz et al., 1979).

Modification of Glycoproteins on Intact Cells and on Spectrin-Free Vesicles with Arylamines. (A) Generation of Aldehydes. Erythrocytes or spectrin-free vesicles (hereafter abbreviated as vesicles) were oxidized for 30 min at 4 °C in 0.5 mM $NaIO_4$ (final concentration) in isotonic phosphate-buffered saline (Liao et al., 1973; Gahmberg & Andersson, 1977). The reaction was stopped by adding glycerol to a final concentration of 5–10 mM. Alternatively, aldehydes were generated enzymatically by galactose oxidase. Erythrocytes or vesicles were washed twice in isotonic buffer of pH 7 (20 mM $NaKHPO_4$–140 mM NaCl). Packed cells were resuspended at 33% hematocrit, and 100 μL of packed vesicles was resuspended in 2.5 mL of buffer containing galactose oxidase (15 units/mL of suspension or as indicated). Samples were incubated for 1 h at room temperature. The conditions chosen for galactose oxidation provided a capacity to oxidize 486 μmol of galactose within 60 min. If the fractional content of galactose residues in band 3 is considered, a total of 4×10^{-8} mol of galactose/mL of suspension had to be oxidized. The capacity of galactose oxidase was therefore in large excess ($\sim 10^4$-fold).

(B) Labeling with [^{14}C]Arylamines. Oxidized cells or vesicles were washed 3 times in an isotonic buffer that had the same pH as the buffer used for modification with [^{14}C]-arylamines. Erythrocytes were labeled at 33% hematocrit. Vesicles were labeled at a concentration of band 3 similar to that determined in the erythrocyte suspension at 33% hematocrit. Oxidized cells or vesicles were resuspended in 1 mL of buffer (20 mM $NaKHPO_4$–50 mM NaCl–120 mM mannitol–30 mM $NaCNBH_3$, pH 6.0) containing 15–30 μCi of either [^{14}C]aniline or [^{14}C]arylalkyldiamine at the concentrations listed. Samples were incubated for 3 h at 4 °C, if not otherwise indicated. Modifications were stopped by adding [^{12}C]aniline to a final concentration of 2 mM to saturate unreacted aldehydes. Following an additional incubation for 15 min, erythrocytes or vesicles were washed 4 times in 30–60 volumes of isotonic phosphate-buffered saline. Modification is shown in microcuries per milligram of membrane protein in order to visualize the degree of possible labeling; corresponding nanomoles are given in the text and Table I.

Preparation of Membranes and Their Characterization. Following modification, cells were lysed at 4 °C and membranes were washed 3 times in 5 mM $NaKHPO_4$–1 mM EDTA (pH 7.4). Samples were removed to analyze for protein and radioactivity. Radioactivity was measured in a Philipps liquid scintillation analyzer. Samples were solubilized in 0.4 mL of NCS tissue solubilizer in glass vials and dissolved in PPO–POPOP–toluene containing 20% ethanol. The efficiency for ^{14}C counting was 97%. Protein was determined on membranes according to standard techniques using bovine serum albumin as a standard. Samples solubilized in $NaDodSO_4$ were frozen at −20 °C until further analysis.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ was performed as previously described (Neville, 1971) by using 8% acrylamide for running and 3% for stacking gels. Samples were denatured with $NaDodSO_4$ in stacking gel buffer for 30 min at 37 °C in the absence of reducing agent if not otherwise indicated. After electrophoresis, gels were stained with Coomassie brilliant blue R 250 and destained in 10% acetic

[1] Abbreviations: arylalkyldiamine, 2-(4-aminophenyl)[1-^{14}C]ethylamine; CuP, copper phenanthroline; Me_2SO, dimethyl sulfoxide; DTBP, dimethyl 3,3′-dithiobis(propionimidate); DTSP, disuccinimidyl 3,3′-dithiobis(propionate); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; f.c., final concentration; NEM, *N*-ethylmaleimide; PAS, periodic acid–Schiff base (stain); POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; $NaDodSO_4$, sodium dodecyl sulfate.

acid containing 20% methanol. The gels were photographed, treated with Me_2SO–PPO as described (Bonner & Laskey, 1974), and vacuum-dried. Dried gels were exposed at −70 °C for 1–7 days to Kodak X-Omat R film.

Extent of Labeling following Modification. The extent of modification of intact cells was determined for both [^{14}C]-aniline and [^{14}C]arylalkyldiamine and was referred to the protein concentration determined on isolated membranes. Computation of incorporated label per milligram of membrane protein was no longer possible for spectrin-free vesicles that contain hemoglobin. A specific labeling in nanomoles of arylamine per nanomole of glycoprotein (band 3 or glycophorin) was determined: Incorporated label and the content of band 3 in modified vesicles and cells were quantified by applying known volumes of vesicles and isolated membranes to $NaDodSO_4$–polyacrylamide gel electrophoresis. Slices of unstained gels were counted for radioactivity. Identical gels were stained with Coomassie blue and scanned by a densitometer (Integraph Bender + Hobein, Zurich). The content of band 3 in vesicles and membranes was determined by reference to a scan from a known amount of total protein from isolated membranes. The amount of label coinciding with band 3 was expressed in nanomoles of incorporated label and referred to nanomoles of band 3, if a M_r of 90 000 is assumed for band 3 (Steck, 1974). The corresponding values for glycophorin A were obtained from nanomoles of incorporated label by assuming that 82% of the protein-bound label was in glycophorin A (Owens et al., 1980). This extent of labeling was referred to the content of glycophorin A as being half of that determined for band 3. These determinations provided the information needed to carry out cross-linking experiments on cells and vesicles at similar degrees of specific labeling.

Cross-linking experiments were carried out at a comparable total concentration of labeled and unlabeled band 3 determined as above. The total concentrations of band 3 in suspensions subjected to cross-linking were (2.4–3.3) × 10^{-6} M for vesicles and 4.2 × 10^{-6} M for erythrocytes.

Cross-Linking with DTSP. (*A*) *On Glycophorin-Containing Vesicles.* Cross-linking of proteins on glycophorin-containing vesicles was performed according to Lomant & Fairbanks (1976). Glycophorin-containing vesicles were resuspended in 20 mM $NaKHPO_4$–100 mM NaCl (pH 8) and cross-linked. Triton extracts that were subjected to cross-linking were first dialyzed against the same buffer containing 0.15% Triton X-100. For cross-linking, 1 volume of vesicle suspension or solubilized protein (at 4 °C) was mixed with an equal volume of 10^{-3} M DTSP in 20 mM $NaKHPO_4$–100 mM NaCl (pH 8) [DTSP was solubilized with dimethyl sulfoxide (f.c. 1%) and mixed with buffer kept at 37 °C]. The reaction mixture was agitated in a Vortex shaker at 4 °C for the indicated times. The cross-linking reaction was stopped by adding lysine to a final concentration of 50–80 mM (pH 8). In controls, 50 mM (f.c.) lysine was added prior to DTSP.

(*B*) *On Spectrin-Free Vesicles and on Intact Erythrocytes.* Cross-linking with DTSP was performed as described above, except that the buffer for cross-linking was isotonic (20 mM $NaKHPO_4$–130 mM NaCl, pH 8) and erythrocytes were cross-linked at 25% hematocrit. *Vesicles* and *cells* were washed 3 times and preincubated for 15 min in cross-linking buffer for pH equilibration. After cross-linking was terminated, intact cells were washed in isotonic buffer. Cells were hemolyzed and washed 3 times in 5 mM $NaKHPO_4$–1 mM EDTA (pH 7.4). Isolated membranes and vesicles were solubilized by adding $NaDodSO_4$ to 1% and NEM to 5 mM (f.c.). Aliquots of the solubilized material were removed for ^{14}C counting and $NaDodSO_4$–polyacrylamide gel electrophoresis.

(*C*) *On Isolated Membranes.* Cross-linking was performed as above in 5 mM $NaKHPO_4$ (pH 8) at a protein concentration of 1 mg/mL. Cross-linked membranes were washed twice in 5 mM $NaKHPO_4$–1 mM EDTA and 1 mM lysine (pH 7.4) and once without lysine. Membranes were solubilized and aliquots removed as mentioned above.

Cross-Linking with Copper Phenanthroline. Cross-linking with copper phenanthroline on isolated membranes was performed according to Steck (1972). A solution of 40 μM $CuSO_4$ and 200 μM *o*-phenanthroline (CuP) was mixed with an equal volume of membranes at a final protein concentration of 1 mg/mL. Cross-linking reactions lasted for 30 min at room temperature at pH 8.0 and were stopped by adding EDTA to a final concentration of 2 mM. For controls, EDTA was added before CuP. Following cross-linking, membranes were washed twice in 5 mM $NaKHPO_4$–1 mM EDTA (pH 7.4).

Quantitation of Cross-Linking. The extent of cross-linking of labeled proteins was quantified from label found in the high molecular weight region of $NaDodSO_4$ gels run without reduction. In brief, dried gels were cut out in lanes and these into pieces according to corresponding X-ray films as shown in Figure 5. The radioactivity bound in the stacking gel region (x) plus that in a high molecular weight region above PAS_1 for glycophorin-modified samples or above band 3 for band 3 modified samples (y) was expressed in percent of the total label recovered in all gel regions (x + y + z) except for the lipids under the tracking dye. These values were corrected by subtraction of corresponding values obtained from controls in which lysine was added before DTSP. The corrected values are given as percent of label cross-linked. The recovery of radioactivity of labeled glycophorins from 8% gels (after Coomassie blue staining and Me_2SO–PPO treatment) was 93.1 ± 3.6% (n = 36), if the following protocol was used for sample preparation: the pieces were further cut into small strips of about 1 × 10 mm size and transferred into glass vials and rehydrated with 0.25 mL of water for at least 4 h; subsequently, 0.75 mL of NCS–water (9:1 v/v) was added and the samples were stored at room temperature for another 15 or 5 h at 40 °C, before adding of 15 mL of PPO–POPOP–toluene containing 20% (v/v) ethanol. Within 6 h, gel pieces swelled to the original volume and became colorless.

Accessibility of DTSP to Surface Proteins. In order to determine whether DTSP added to arylamine-modified erythrocytes remained accessible to surface components, the cross-linking capacity in an erythrocyte suspension was monitored by studying cross-linking of exogenously added soluble protein (20 μM hemoglobin, final concentration). Cross-linking with cells at 25% hematocrit was initiated as above and hemoglobin was added at the given times. Controls were run in the absence of erythrocytes. Cross-linking was stopped 8 min after the addition of hemoglobin. Cells were pelleted and the supernates were analyzed for cross-linked hemoglobin by electrophoresis under nonreducing conditions on 12% $NaDodSO_4$–polyacrylamide gel electrophoresis. Cross-linked hemoglobin was quantified by densitometry of dried gels as above. Alternatively, hemoglobin was [^{125}I]iodinated for 1 min with Chloramine T (Bächi et al., 1977) and used in identical experiments as above and in experiments to determine the amount of hemoglobin which was cross-linked to intact erythrocytes. To correct for cell-bound, but not cross-linked, hemoglobin, we ran controls in the presence of 50 mM lysine. After the reactions were terminated by lysine, cells were washed 4–5 times in 40 volumes of buffer prior to determi-

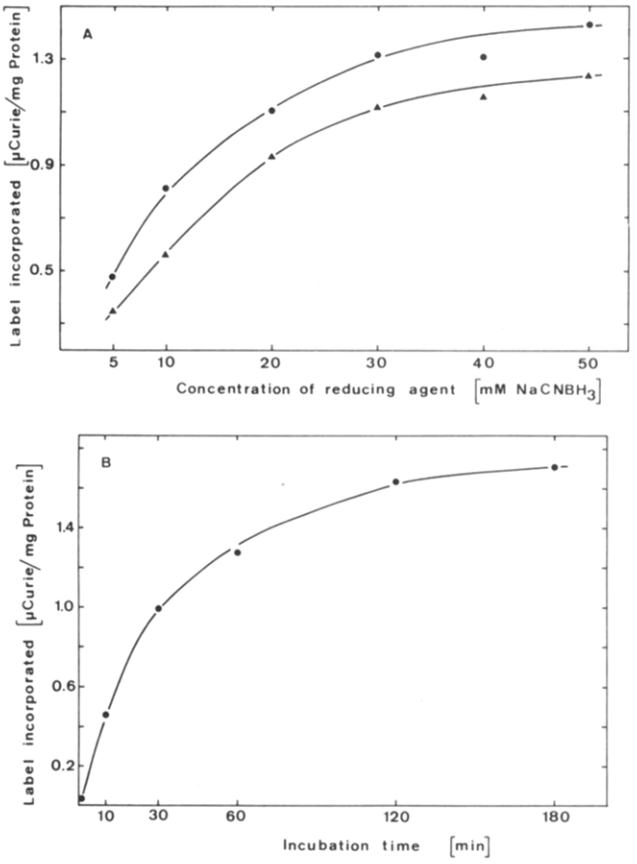

FIGURE 1: Effect of $NaCNBH_3$ concentration and temperature on the extent of [^{14}C]arylamine modification. Packed, periodate-oxidized, and washed erythrocytes (0.97 mL) were incubated with 15 μCi of [^{14}C]aniline in 1.03 mL of buffer of pH 6.5. The buffers consisted of 20 mM $NaKHPO_4$, 50 mM NaCl and varying concentrations of mannitol and $NaCNBH_3$. All buffer solutions had an osmolarity of 325 mosM. The concentration of [^{14}C]aniline was 0.076 mM. The reaction mixtures were incubated for 2 h and the reactions were terminated as described under Materials and Methods. (●) 37 °C; (▲) 4 °C. (B) Time course of modification. Erythrocytes were modified with [^{14}C]arylalkyldiamine as described in (A) with 30 mM $NaCNBH_3$. Samples were removed at the indicated times and analyzed as described. Controls for nonoxidized cells were incubated for 30 s, 10 min, and 180 min, respectively. All three samples showed an incorporation of less than 2 nCi/mg of membrane protein.

nation of bound label. Cross-linked label to intact erythrocytes is expressed in nanomoles of hemoglobin per milliliter of packed erythrocytes and represents the value obtained after subtraction of controls.

Results

Amino Group Supplementation of Glycoproteins on Intact Cells. Modification of intact human red blood cells with [^{14}C]arylamines was carried out by Schiff base formation with aldehyde groups generated on cells by chemical or enzymatic oxidation. Substantial modification of oxidized cells required reduction of the imine with $NaCNBH_3$ (Figure 1). The optimal $NaCNBH_3$ concentration was between 30 and 50 mM. Raising the temperature from 4 to 37 °C increased the amount of incorporated label only slightly. Incorporation of [^{14}C]-arylalkyldiamine showed saturation after 2 h of modification with label and $NaCNBH_3$ (Figure 1).

The type of oxidation to generate aldehyde groups allowed to direct incorporated [^{14}C]aniline or [^{14}C]arylalkyldiamine preferentially into certain proteins as previously reported for borohydride reduction (Gahmberg & Andersson, 1977). While a chemical oxidation with periodate directs labeled arylamines exclusively to sialoglycoproteins, enzymatic oxidation results in preferential labeling of band 3 protein, the band 4.5 region, and the lipids (Figure 2). Treatment of cells with neuraminidase before galactose oxidation directs label primarily to band 3, the sialoglycoproteins, and the lipids. Extensive galactose oxidation directs label also to the band 4.5 region representing polyglycosyl conjugates (Krusius et al., 1978; Dejter-Juszynski et al., 1978; Childs et al., 1979; Koscielak et al., 1979). Modification with arylalkyldiamine increases the positive charge of modified glycoproteins considerably, because the introduced alkylamino groups (pK = 10.5) are protonized at the pH of the running gel (pH 8.8). This resulted in a slightly reduced electrophoretic mobility of the heavily modified glycophorin A (PAS_1 and PAS_2, Figure 2) when compared to that of aniline-modified glycophorin A. Except for this, both aniline- and arylalkyldiamine-modified erythrocytes reveal essentially the same labeling pattern (Figure 2).

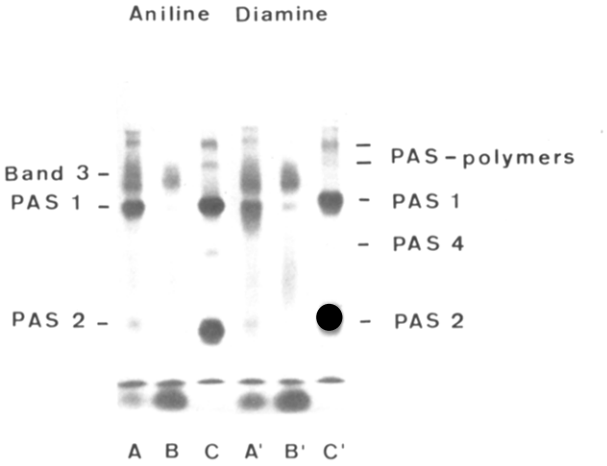

FIGURE 2: Fluorograph of a polyacrylamide gel run with membranes from [^{14}C]arylamine-modified erythrocytes. Erythrocytes were oxidized with periodate or with galactose oxidase without or after neuramindase treatment. For pretreatment with neuraminidase, 20 milliunits of enzyme and 1 mL of packed erythrocytes were resuspended in 2 mL of 20 mM $NaKHPO_4$, 130 mM NaCl, 5 mM $CaCl_2$, and 10 mM glucose (pH 6.0). The suspension was incubated for 30 min at 37 °C. Erythrocytes were washed twice in isotonic buffer of pH 7 for subsequent treatment with galactose oxidase. Galactose oxidase treatment was carried out with 15 units/mL of packed cells and modification with 15 μCi of either [^{14}C]aniline (0.076 mM) or [^{14}C]arylalkyldiamine (0.129 mM). Modified erythrocytes were washed and membranes isolated. Equal amounts of radioactively labeled membranes (25 000 cpm) were supplemented with nonlabeled membranes to give equal amounts of protein (22 μg) prior to denaturation with $NaDodSO_4$ and 40 mM DTT. Intact cells were treated with neuramindase/galactose oxidase (A, A′), galactose oxidase (B, B′), or periodate (C, C′).

Amino Group Supplementation Occurs via the Arylamino Group. The following experiments were designed to analyze whether only the arylamino group or both the aryl- and alkylamino groups of 2-(4-aminophenyl)ethylamine (abbreviated as arylalkyldiamine) form imines with aldehydes. The rationale was to compare the incorporation of arylalkyldiamine with that of aniline and phenylethylamine at different pH values. Schiff base formation is favored at low pH values. However, the pH value during modification should not be below pH 6, to avoid pH-dependent protein rearrangements in erythrocyte membranes (Liu et al., 1977). At pH 6–6.5, the alkylamino group is protonized completely, whereas the arylamino group is only weakly protonized and readily reacts with carbonyl groups.

As expected, the amount of incorporated, labeled aniline and arylalkyldiamine increased steeply with decreasing pH values from 7.5 to 6 and reached 1–2.5 μCi/mg of membrane

Table I: Modification of Intact Human Erythrocytes with [U-^{14}C]Aniline, Phenyl[1-^{14}C]ethylamine, and 2-(4-Aminophenyl)[1-^{14}C]ethylamine[a]

conditions of modification			incorporation of label at pH 6–6.4 (nmol/mg of membrane protein)		
oxidation with	addition of $NaCNBH_3$	μCi of label added/mL of packed cells	alkylamine	arylamine	arylalkyldiamine
periodate	+	15	0.13 ± 0.004 (n = 3)	10.4	31 ± 2.8 (n = 5)
periodate	–	15	n.d.	n.d.	0.16
–	+	15	0.008	0.21	0.05 ± 0.01 (n = 3)
periodate	+	25	n.d.	13.1	30.3
GaO[b]	+	25	n.d.	10	9.9

[a] For experimental details see the legend of figure 2. [b] Erythrocytes were treated with galactose oxidase and modified for 3 h at 4 °C.

protein at pH 6 (Figure 3). On the contrary, the extent of incorporated phenylethylamine remained very low (2–6 nCi/mg of membrane protein) within the entire pH range tested (note the change of scale in Figure 3). Thus, the arylamino group was at least 280 times more reactive than the alkylamino group at pH 6–6.5 (Table I).

Extent of Amino Group Supplementation on Intact Erythrocytes. Table I gives a comparison of the degree of modification with all labels applied at the optimal pH value. Incorporation of both [^{14}C]aniline and [^{14}C]arylalkyldiamine was strictly dependent on oxidation. Omission of the reducing agent $NaCNBH_3$ resulted in negligible amounts of covalently bound arylalkyldiamine. Modification of periodate oxidized cells by arylalkyldiamine resulted in 31 nmol of label/mg of membrane protein (Table I). This corresponds to approximately 18 ± 3 (n = 7) additional alkylamino groups per copy of glycophorin, as determined on the basis that 80% of the label is in glycophorin A (obtained from the distribution of radioactivity within the gel) of which 5 × 10^5 copies are present per cell (Fairbanks et al., 1971). Modification of galactose oxidase treated cells for 3 h showed saturation for both [^{14}C]aniline and [^{14}C]arylalkyldiamine (Table I). Following this treatment, 35% of incorporated label is found in band 3. Assuming 10^6 copies of band 3 per cell (Fairbanks et al., 1971), the extent of modification with [^{14}C]arylamines corresponds to 0.42 ± 0.07 (n = 4) mol/mol of band 3 on intact erythrocytes.

Amino Group Supplementation Enhances Cross-Linking of Glycophorins on Glycophorin-Containing Vesicles. We asked whether supplementation of glycoproteins with additional amino groups increases their cross-linking probability when studied with amino group specific cross-linking reagents on solubilized glycophorins and on reconstituted vesicles containing glycophorins (Lutz et al., 1979). These model systems differ considerably from intact cells since they lack cytoskeletal restraints. Cross-linking of glycophorins obtained with DTSP is due to existing interactions as well as collisions of proteins within the plane of the membrane and to some extent to collisions of vesicles.

Glycophorins were labeled in intact erythrocytes with arylalkyldiamine or with aniline. Aniline-modified glycophorins served as controls. Glycophorins were extracted and glycophorin-containing vesicles were reconstituted with coextracted lipids by detergent removal; 67 ± 6% of glycophorins are normally oriented in these glycophorin-containing vesicles (Lutz et al., 1979). The highest concentration of cross-linker used was 0.5 mM in order to prevent excess monofunctional modifications. This concentration exceeds that of the introduced free amino groups by a factor of roughly 5.

Glycophorins supplemented with additional amino groups display an elevated degree of cross-linking compared to that of aniline-modified glycophorins both in detergent-solubilized

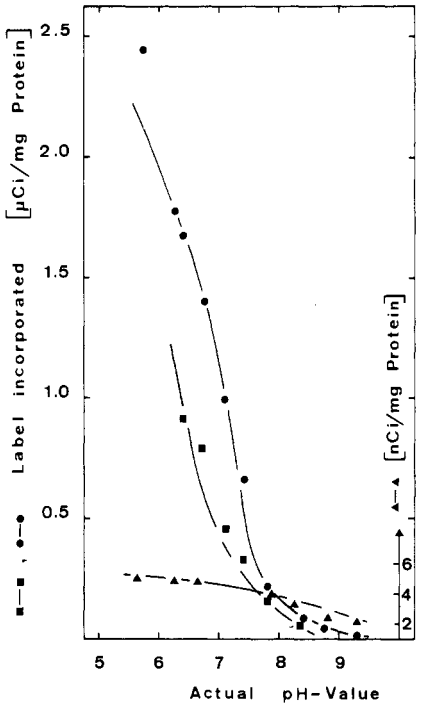

FIGURE 3: pH dependency of modification with [^{14}C]aniline, [^{14}C]phenylethylamine, and 2-(4-aminophenyl)[1-^{14}C]ethylamine. After oxidation with periodate, erythrocytes were equilibrated twice in the appropriate buffer for 15 min at 4 °C. One milliliter of packed cells was resuspended with an equal volume of the appropriate buffer solution containing 30 mM $NaCNBH_3$. Buffers were for pH 5.5 (20 mM sodium acetate, 65 mM NaCl, and 100 mM mannitol), for pH 6 to 7.5 (20 mM $NaKHPO_4$, 50 mM NaCl, and 120 mM mannitol), for pH 8 to 9.5 (20 mM borate and varying concentrations of NaCl to obtain an osmolarity of 325 mosM). Labeled amines (15 μCi) were added. The concentrations differed, because of the different specific activities, and were 0.076 mM for aniline, 0.129 mM for arylalkyldiamine, and 0.155 mM for phenylethylamine. All samples were incubated for 2 h at 4 °C. The pH values in the suspensions were measured before and after the incubation and the mean was taken as actual pH value. After incubation, the cells were spun down and analyzed as described. The first supernatant fluid was collected and the OD_{420} was determined as a measure for the degree of hemolysis. In the pH range from 5.7 to 7.3 OD_{420} values were constant (1.5) and increased to 5 at pH 9.3. (●) Arylalkyldiamine; (■) aniline; (▲) phenylethylamine.

glycophorins and in glycophorin-containing vesicles (Figure 4). Introduction of arylalkyldiamine increases the rate of cross-linking compared to that obtained with aniline-modified vesicles. Cross-linking reaches 38% for arylalkyldiamine and 26% for aniline-modified glycophorins when analyzed on reconstituted vesicles. Cross-linking of detergent-solubilized glycophorins is low compared to that observed in reconstituted vesicles (Figure 4). This could be due to hydrophobic interactions of the cross-linker with the detergent which reduce the

Table II: Cross-Linking of Glycoproteins on Intact Human Erythrocytes[a]

preferentially labeled glycoprotein[b]	% of label cross-linked[c] on cells modified with [^{14}C]aniline (control)	[^{14}C]aryl-alkyldiamine (amino group supplemented)	significance, t test,[d] P
glycophorins	0.9 ± 0.1 ($n = 6$)[e]	1.8 ± 0.6 ($n = 6$)[e]	<0.02
band 3	0.8 ± 0.6 ($n = 4$)[f]	1.7 ± 0.9 ($n = 7$)[g]	<0.02

[a] For details see Materials and Methods. [b] Incorporation of label was directed into glycophorins or band 3 by the type of oxidation. [c] The mean values and standard deviations are given. [d] Confidence level by which control and amino group supplemented samples differ. [e] Data were derived from three experiments with six measurements. [f] Three experiments and four measurements. [g] Four experiments and seven measurements.

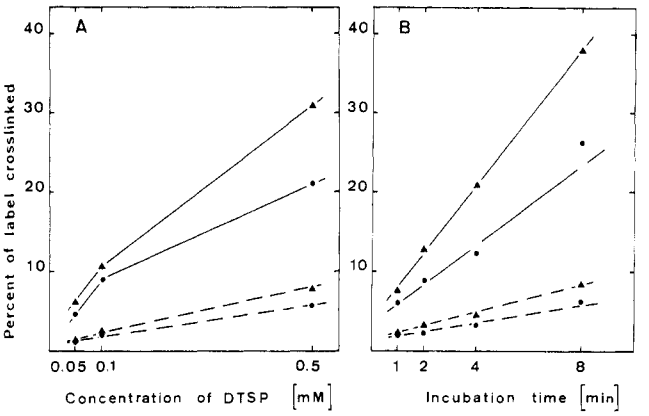

FIGURE 4: Effect of DTSP concentration (A) and incubation time (B) on the extent of cross-linking of [^{14}C]arylamine-modified glycophorins in glycophorin-containing vesicles and in extracts. Labeled glycophorin from ^{14}C-labeled modified intact erythrocytes was either reconstituted in vesicles or solubilized in Triton X-100. Fifty micrograms of labeled glycophorin in either form in 100 μL of buffer was cross-linked for each point and analyzed as described under Materials and Methods. (—) Glycophorin-containing vesicles; (- - -) solubilized glycophorin; (●) aniline-modified glycophorins; (▲) arylalkyldiamine-modified glycophorins.

concentration of reactive reagent.

While the quantitative data demonstrate increased rates of cross-linking for amino group supplemented glycophorins in vesicles, the qualitative data (Figure 5) depict differences in the early cross-linking events between aniline-and arylalkyldiamine-modified glycophorins. Two minor bands, indicated by arrows (a, b) represent cross-linked species containing glycophorins that participate in cross-linking primarily in arylalkyldiamine-modified vesicles. In vesicles containing aniline-modified glycophorins, band b does not seem to undergo further cross-links and band a is immediately cross-linked to higher molecular weight. These selective changes demonstrate an advantage of the introduced alkylamino groups in allowing generation of sufficient amounts of definable oligomeric complexes prior to the formation of multimeric complexes. Since DTSP is a cleavable reagent, reduction of samples at 37 °C prior to electrophoresis mostly reverses the cross-linking effect (Figure 5B). Complete reversibility was observed following reduction for 3 min at 100 °C (not shown). These findings illustrate that amino group supplementation is a powerful tool to study cross-linking of glycoproteins.

Cross-Linking of Glycoproteins on Intact Red Blood Cells and Isolated Membranes. Amino group supplementation allows one to evaluate whether a minute extent of cross-linking of a certain protein is due to a lack of endogenous amino groups or is an inherent property of the protein arrangement as shown here for intact erythrocytes. Cross-linking of proteins on intact cells was studied on both aniline and arylalkyldiamine-modified cells. Cross-linking on band 3 modified and amino group supplemented erythrocytes resulted in little label at the start of the running gel (Figure 6E) as compared to control samples in which the reaction was quenched with lysine (Figure 6F) or in which cross-linked proteins were reduced (Figure 6E′). A quantitation of the extent of cross-linking reveals less than 2% of the total label as cross-linked species for either glycophorin-modified or band 3 modified, amino group supplemented erythrocytes (Table II). Considering the relatively large standard deviations, amino group supplemented erythrocytes show a 2-fold enhancement over aniline-modified samples. A similar enhancement of cross-linking by amino group supplementation was also found, when young and senescent erythrocytes were studied (Table III). However, cross-linking of glycoproteins was slightly increased (by 0.4% of the total labeled protein) on senescent erythrocytes. The two means are different at a confidence level of 0.06. The exclusive detectability of this difference on amino group supplemented samples demonstrates that cross-linking was via introduced arylalkyldiamine. Nevertheless, erythrocytes of any cell age show very little cross-linkability of band 3 and glycophorin at 0–4 °C even after amino group supplementation (Figure 6F). It is important to add that cross-linking of band 3 was extensive, as found previously (Steck 1972; Wang & Richards, 1974; Staros et al., 1974), when analyzed on isolated membranes either by copper phenanthroline (Figure 6A) or by DTSP (Figure 6C). Both types of cross-linking patterns were prevented by addition of EDTA in the case of copper phenanthroline (Figure 6B) and lysine when DTSP was used (Figure 6D). Reductive cleavage of cross-links (Figure 6A′,C′) was substantial when compared with cross-linked samples

Table III: Cross-Linking of Glycoproteins on Erythrocyte Subpopulations[a]

erythrocyte subpopulation	rel creatine content[b]	rel incorporation of label[c]		% of label cross-linked[d] on cells modified with	
		[^{14}C]aniline	[^{14}C]arylalkyldiamine	[^{14}C]aniline	[^{14}C]arylalkyldiamine
young	5.8 ± 0.8	1.05 ± 0.04	1.07 ± 0.05	0.8 ± 0.3 ($n = 3$)	1.5 ± 0.3 ($n = 4$)[e]
senescent	1	1 ± 0.04	1 ± 0.05	0.9 ± 0.7 ($n = 6$)	1.9 ± 0.3 ($n = 6$)[e]

[a] Human erythrocytes were fractionated into subpopulations as described under Materials and Methods. Young and senescent erythrocyte subpopulations were oxidized with galactose oxidase before labeling. [b] The creatine content of erythrocytes in micrograms per 10^{10} cells served as the cell-age parameter. A ratio of the creatine contents of young over that of senescent cells is given (average from three independent experiments). [c] The relative incorporation of label was determined from incorporated label per milligrams of membrane protein in three independent experiments and six protein determinations in each. The degree of modification as studied in these experiments was varied from 7.1 to 12.8 nmol of label incorporated/mg of membrane protein. [d] Data were derived from two independent experiments with n (numbers) measurements. Mean values and standard deviations are given. [e] The values of cross-linked labeled material in arylalkyldiamine-modified young and senescent erythrocytes are different at a confidence level of 0.06.

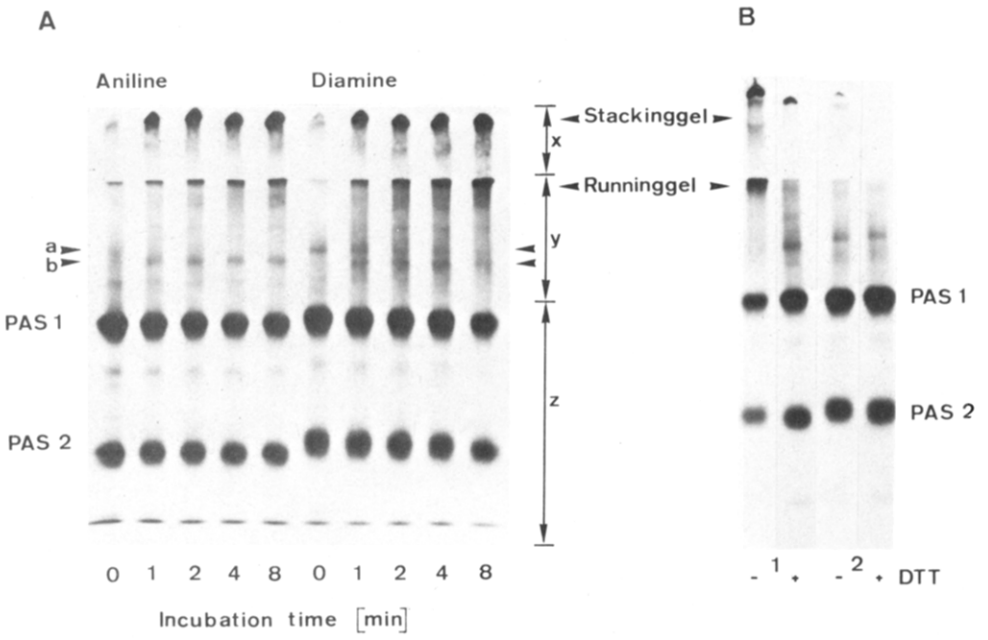

FIGURE 5: Fluorograph of a polyacrylamide gel run with cross-linked samples of glycophorin-containing vesicles. Unreduced samples were run on a 8% gel. (A) Time course of cross-linking as described in Figure 4. Beside cross-linked label on top of the stacking gel, the running gel, and the PAS regions, the arrows a and b indicate polymers that appear (b) or disappear (a) during this time course. (O) Control sample; lysine was added prior to DTSP. Quantitation of cross-linking, as shown in Figure 4, is based on the gel regions indicated as x, y, and z and is described under Materials and Methods. (B) Cleavability of cross-linked products. Samples were denatured with 1% $NaDodSO_4$ for 30 min at 37 °C in the presence (+) or absence (−) of 40 mM DTT prior to electrophoresis. (1) Cross-linked glycophorins; (2) controls.

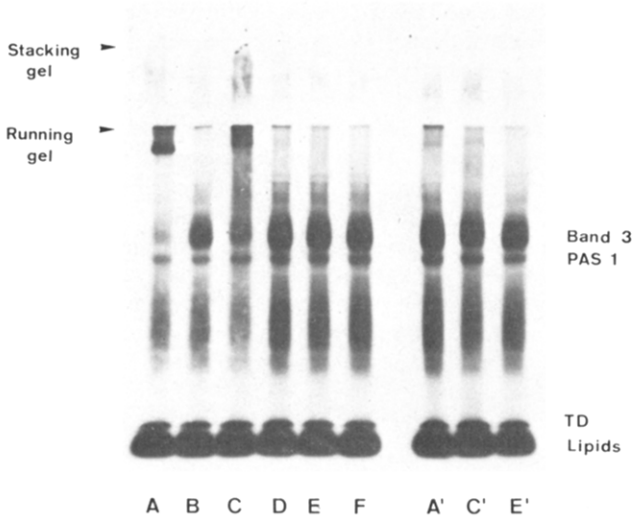

FIGURE 6: Fluorograph of a polyacrylamide gel run with membranes from CuP and DTSP cross-linked glycoproteins obtained by cross-linking of isolated membranes and of intact erythrocytes. Band 3 modified intact erythrocytes, labeled with [^{14}C]arylalkyldiamine, were either used as such for cross-linking or following membrane isolation. Cross-linking was analyzed at comparable concentrations of membrane protein by using erythrocytes at 25% hematocrit and isolated membranes at 1 mg/mL. Cross-linking with CuP (A) was carried out on isolated membranes as outlined. In control samples (B), EDTA was added prior to CuP. Cross-linking with 0.5 mM DTSP was carried out for 8 min at 0–4 °C either on isolated membranes (C, D) or on intact cells (E, F) and corresponding controls (D, F) contained 50 mM lysine. After termination of the reactions, erythrocytes or membranes were washed, denatured, and electrophoresed. Samples A–F were electrophoresed without reduction, whereas those in A′–E′ following reduction with 40 mM DTT. (A) Isolated membranes cross-linked with CuP; (B) corresponding control; (C) isolated membranes cross-linked with DTSP; (D) corresponding control; (E) intact erythrocytes cross-linked with DTSP; (F) corresponding control: (A′, C′, and E′) as in (A), (C), and (E) but electrophoresed after reduction.

(Figure 6A,C) but incomplete when compared with reduced samples from membranes of DTSP-treated cells (Figure 6E′).

Table IV: Cross-Linking of Proteins on Isolated Membranes[a]

preferentially labeled glycoprotein	cross-linking reagent	% of label cross-linked[b] [^{14}C]aniline	% of label cross-linked[b] [^{14}C]arylalkyldiamine
glycophorins	DTSP	1.7	2.8
	CuP	3.8	3.1
band 3	DTSP	23.7	36
	CuP	26.4	30.5

[a] Membranes were isolated from [^{14}C]arylamine-labeled erythrocytes. For details, see Materials and Methods. [b] Data give the average from two experiments.

These data demonstrate an unexpected difference in the cross-linking probability of band 3 on isolated membranes and intact cells (Figure 6C,E).

Quantitation indicates that cross-linking of glycoproteins in membranes isolated from band 3 modified cells is roughly 20-fold higher than on intact cells (Table IV). This difference is not simply due to the fact that both sides of the membrane were accessible to the cross-linker, because cross-linking of glycoproteins with DTSP on isolated membranes of amino group supplemented cells yet displayed a significantly higher extent (1.5-fold) than that in membranes from aniline-modified control samples. While cross-linking of band 3 and polyglycosyl conjugates was greatly increased following cell lysis, cross-linking of glycophorins was only 2–3 times higher in isolated membranes than in intact cells. The 2–3-fold increase in cross-linked glycophorins was independent of amino group supplementation. These results suggest that the membrane undergoes a dramatic structural alteration during cell lysis which affects band 3 and glycophorins differently.

The low extent of cross-linking observed on intact cells contrasts with that on isolated membranes. Though cells and isolated membranes were analyzed at similar concentrations of band 3 in suspension (see Materials and Methods), the concentration of DTSP-reactive groups within these structures differed. Thus, we analyzed whether DTSP added to eryth-

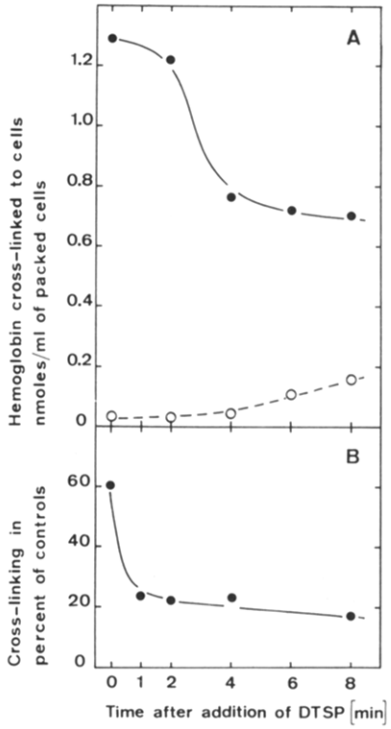

FIGURE 7: Accessibility of DTSP to erythrocyte surface proteins. Arylalkyldiamine-modified erythrocytes were cross-linked at 25% hematocrit and hemoglobin (20 μM, f.c.) was added at the indicated times to these suspensions. Cross-linking of hemoglobin in solution and cross-linking of soluble hemoglobin to intact cells were determined as outlined under Materials and Methods. Cross-linking of radiolabeled hemoglobin to intact cells is given in nanomoles hemoglobin per milliliter of packed cells in (A). Cross-linking of hemoglobin in solution is given in percent (average of two experiments) of that observed, if hemoglobin alone was added to DTSP at the given times (B). In these controls, 13.4 nmol/mL hemoglobin was found in cross-linked oligomers when added immediately to DTSP and 12.8 nmol when added to DTSP that was kept for 8 min at 4 °C. (*A*) (●) Hemoglobin cross-linked to intact cells at 4 °C; (○) hemoglobin cross-linked to intact cells at room temperature. (B) Cross-linking capacity in a mixture of erythrocytes and exogenously added hemoglobin as measured on hemoglobin in solution.

rocytes was trapped by cytoplasmic proteins or remained accessible to surface proteins. We found that exogenous hemoglobin added to a mixture of DTSP and arylalkyldiamine-modified erythrocytes at 25% hematocrit was cross-linked to intact cells [1.3 nmol/mL of packed cells (Figure 7A)]. When added after erythrocytes reacted with DTSP for 8 min, the amount of hemoglobin cross-linked to erythrocytes was half of that measured when hemoglobin and erythrocytes were added simultaneously to DTSP. Note, however, that the capacity to cross-link hemoglobin to intact erythrocytes was greatly decreased at higher temperature (Figure 7A). The accessibility of DTSP to added hemoglobin in solution was initially 60% of that found when hemoglobin alone was cross-linked and dropped during cross-linking in the presence of erythrocytes to 16% (Figure 7B). These results indicate that sufficient DTSP was available for cross-linking of surface proteins. Hence, the minute extent of cross-linking on intact cells cannot be attributed to a cytoplasmic trapping of DTSP. It is also not due to a lack of accessible amino groups as shown by a comparison of cross-linking on aniline- and arylalkyldiamine-modified cells. Therefore, it is indicative of an inherent property of the membrane structure of intact erythrocytes.

Cross-Linking of Glycoproteins on Spectrin-Free Vesicles. In order to investigate whether the low extent of cross-linking

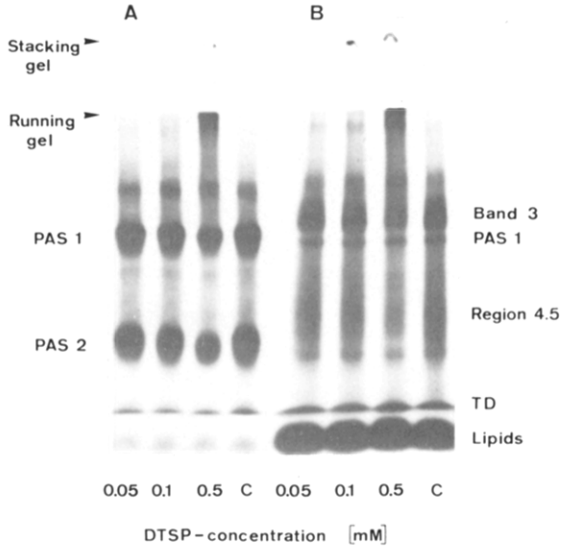

FIGURE 8: Fluorograph of a polyacrylamide gel run with cross-linked glycoproteins from spectrin-free vesicles, modified with arylalkyldiamine after periodate oxidation (A) and after galactose oxidase treatment (B). In controls (C) 80 mM lysine was added before DTSP (0.5 mM). Cross-linking lasted for 8 min at 0–4 °C and was stopped by adding 80 mM lysine. TD indicates the tracking dye region.

on intact cells in fact originated from structural aspects of the membrane, we studied glycoprotein cross-linking on spectrin-free vesicles. These vesicles are released spontaneously from ATP-depleted human erythrocytes (Lutz et al., 1977b). Their content of band 3 and glycophorins remains the same per surface area as on intact cells (Lutz et al., 1977b; Lutz, 1978; Müller et al., 1981). They contain hemoglobin but lack the cytoskeleton and hence the restraints imposed on transmembrane proteins. These surface properties make spectrin-free vesicles the closest model system for intact erythrocytes except for the protein mobility. DTSP-dependent cross-linking was therefore studied on these vesicles following incorporation of [^{14}C]aniline or [^{14}C]arylalkyldiamine into glycophorins by a chemical generation of aldehydes (glycophorin-modified vesicles) or into band 3, polyglycosyl conjugates, and glycolipids by an enzymatic generation of aldehydes (band 3 modified vesicles).

Figure 8 shows a fluorograph comparing the cross-linking patterns obtained with increasing concentrations of DTSP with glycophorin-modified vesicles (A) and band 3 modified vesicles (B). The fraction of label detectable at the start of the running gel increased with DTSP concentration in both preparations. Cross-linked material in glycophorin-modified vesicles involved PAS_1 and PAS_2 as evident from a decrease in the labeled PAS_1 and PAS_2 region at 0.5 mM DTSP. Similarly, label in band 3 and the region 4.5, probably consisting of polyglycosyl conjugates (Krusius et al., 1978), was reduced in content following cross-linking on band 3 modified vesicles. Two-dimensional gel electrophoresis using reductive cleavage in the second dimension showed these components as undergoing cross-linking, with band 3 being the most prevalent one (not shown).

Cross-linking on vesicles was subsequently analyzed on vesicles modified with [^{14}C]aniline or [^{14}C]arylalkyldiamine to study the effect of amino group supplementation. A quantitation of the extent of cross-linking obtained with control and amino group supplemented samples is shown in Figure 9. Cross-linking on glycophorin-modified vesicles reached 21.8% of the total label within 8 min for amino group supplemented samples, but only 7.5% for control samples (Figure 9A). Cross-linking on band 3 modified vesicles reached 19%

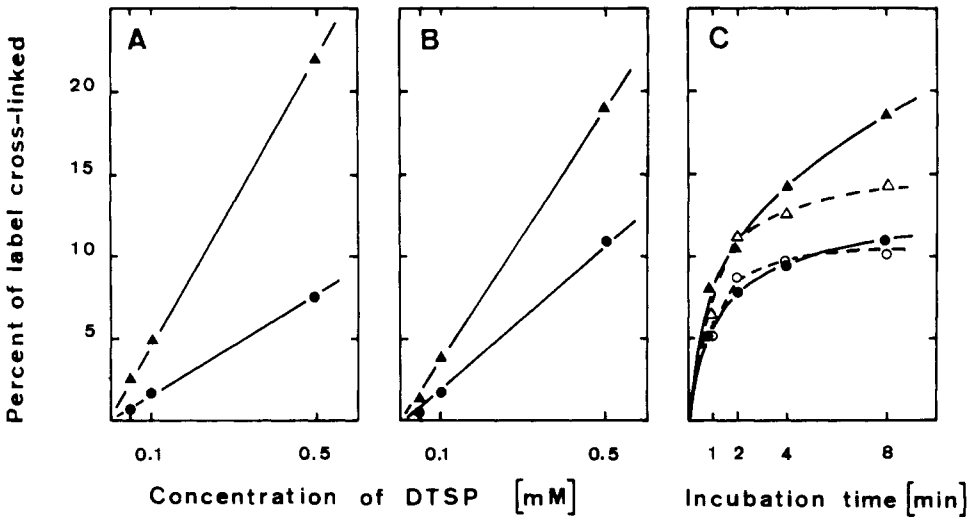

FIGURE 9: Effect of DTSP concentration on cross-linking of glycoproteins on spectrin-free vesicles. Equal amounts of glycophorin-modified (A) or band 3 modified spectrin-free vesicles (B, C) were resuspended in equal volumes of buffer, and aliquots of 100 μL were subjected to cross-linking at 0–4 °C at the concentrations of modified proteins as given under Materials and Methods. The time-dependent cross-linking was analyzed at 0.5 mM DTSP on band 3 modified vesicles that were modified to equal extents with aniline or arylalkyldiamine but contained either 1.3 mol/mol of band 3 (solid line) or 0.5 mol/mol of band 3 (- - -) of label. Cross-linking was stopped by adding lysine after the time indicated (C) or after an incubation for 8 min in (A) and (B). Cross-linking was quantified and is shown in percent of cross-linked label as outlined under Materials and Methods. (A) cross-linking on glycophorin-modified vesicles; (B) cross-linking on band 3 modified vesicles; (C) time course of cross-linking on band 3 modified vesicles. (▲, △) Arylalkyldiamine-modified samples; (●, ○) aniline-modified samples.

of the total label for amino group supplemented samples and 11% for control samples (Figure 9B). These results clearly indicate an enhanced cross-linking of amino group supplemented glycoproteins. The enhancement is slightly smaller with band 3 modified vesicles than with glycophorin-modified vesicles. This is most likely due to the fact that glycophorin-modified vesicles contain approximately 24–27 new amino groups per glycophorin whereas band 3 modified vesicles contained at the most 1–1.3 new amino groups per band 3. In both systems, the enhancement is significant and reveals a faster rate of cross-linking following amino group supplementation of vesicles. The time courses (Figure 9C) are biphasic with both types of label, suggesting that a substantial fraction of cross-links represents preexisting interactions rather than diffusion-controlled formation of new complexes. The extent of glycoprotein cross-linking on vesicles increased with the degree of amino group supplementation but remained unaltered whether vesicles contain 0.5 or 1.3 mol of aniline instead of arylalkyldiamine per mol of band 3.

Discussion

Amino Group Supplementation of Surface Glycoproteins. Supplementation of surface glycoproteins with free alkylamino groups enhances the probability of modified glycoproteins to be cross-linked. The use of a radioactively labeled amine greatly facilitates quantitative and qualitative analysis. The conditions for modification are mild and distinct proteins can be labeled with high selectivity. Only small amounts of radioactive label are necessary to yield a high degree of labeling. This high extent of ^{14}C modification is mainly due to the use of sodium cyanoborohydride as a reducing agent which can be added simultaneously with arylamines, because it does not reduce aldehydes (Lane, 1975). It selectively reduces Schiff bases but does not reduce SS bridges (Jentoft & Dearborn, 1979; Dottavio-Martin & Ravel, 1978). Modification can be performed at low temperatures that minimize temperature-dependent structural alterations in the membrane. Modification following periodate oxidation reached 7.2×10^6 molecules of arylalkyldiamine per cell at pH 6–6.5 under standard conditions. Similar degrees of labeling have been obtained by introducing α-thiomannosyl hydrazides into bovine erythrocytes (1.4×10^6 molecules per cell) (Rando et al., 1979).

It was essential for our work to assure that the alkylamino group of arylalkyldiamine did neither react with aldehydes during modification nor react with reducing oligosaccharides. At higher pH values than used here, Ginsburg et al. showed that alkylamino groups can form Schiff bases with reducing oligosaccharides (Jeffrey et al., 1975; Smith & Ginsburg, 1980). We demonstrate that under the conditions described, Schiff bases are formed almost exclusively with the arylamino group. This conclusion is supported by the following finding: Incorporation of phenyl[1-^{14}C]ethylamine was very low over the entire pH range tested and amounted to 0.12 nmol/mg of membrane protein as compared to that of arylalkyldiamine (31 nmol/mg of membrane protein). Such a low incorporation of alkyldiamine was expected for pH 6–6.5 but not for higher pH values, because protonation prevents it from forming a Schiff base. The low extent of modification at elevated pH values was found in two experiments with nine determinations. This could be due to the pK of the alkylamino group (about 10.5) that may yet require more basic conditions. Alternatively, modification at pH 9 could be inhibited by cyanide (Jentoft & Dearborn, 1980) which is a side product of the reduction. At pH 6–7 no such inhibition by HCN (pK = 9.3) is expected, because the cyanide ion rather than HCN forms adducts with aldehydes.

Modification with labeled arylamines provides a specific labeling of sialoglycoproteins or of galactos-containing glycoproteins depending on the type of aldehyde generation. The labeling specificity obtained is very similar to that found upon periodate oxidation or galactose treatment and reduction with tritiated borohydride (Gahmberg & Andersson, 1977; Gahmberg, 1976). Band 3, polyglycosyl conjugates (Krusius et al., 1978), and lipids are primarily labeled following galactose oxidase treatment. Glycophorins (PAS_1–PAS_4) are the major labeled components following aldehyde generation by periodate. Beside the sialoglycoproteins PAS_{1-4}, small amounts of glycophorin polymers were labeled with aniline and arylalkyldiamine (Figure 2). Since these PAS polymers have earlier been observed on $NaDodSO_4$ gels of erythrocyte

membranes that were not modified via aldehydes and $NaCNBH_3$ (Tuech & Morrison, 1974; Orr, 1981), and since they were labeled with both aniline and arylalkyldiamine to about the same extent, arylalkyldiamine itself does not cross-link proteins in measurable amounts during modification.

Cross-Linking Studies on Amino Group Supplemented Glycoproteins. We demonstrated that amino group supplementation enhanced cross-linking of glycophorins in glycophorin-containing vesicles. This enhancement is independent of the question to what extent DTSP penetrated the vesicle membrane, because penetration of DTSP would occur in both aniline- and arylalkyldiamine-modified samples. By applying this technique to intact cells and to spectrin-free vesicles, we determined a high extent of glycoprotein cross-linking on spectrin-free vesicles (vesicles) but a very small extent on intact erythrocytes at 0–4 °C. At this low temperature, the long-range lateral mobility of mobile integral membrane proteins is no longer detectable on ghosts (Golan & Veatch, 1980) and fused erythrocytes (Fowler & Branton, 1977; Schindler et al., 1980), because of the high lipid viscosity. Hence, the cross-linking conditions chosen here, primarily probe for preexisting protein–protein interactions as evident from a biphasic time course for glycoprotein cross-linking on vesicles. This suggests that vesicles contain preexisting protein–protein interactions and are yet capable of forming new complexes at a slow rate which are cross-linkable via accessible, endogenous amino groups. Amino group supplementation enhances both the initial fast phase and the slow phase of cross-linking. We can assume that aniline- and arylalkyldiamine-modified vesicles show an identical degree of cytoplasmic cross-links of unknown extent. Given the most unlikely case that all cross-links on aniline-modified vesicles occur on the cytoplasmic side of the membrane, the difference in the extent of cross-linking between amino group supplemented and control samples reaches 8% for band 3 modified and 14.3% for glycophorin-modified vesicles. This absolute minimum for exoplasmic cross-linking is only observed with vesicles but not with intact cells for which the corresponding value amounts to 0.9% for both glycophorin and band 3 modified cells. This indicates that the minute degree of cross-linked glycoproteins on intact cells is not due to an inaccessibility of endogenous amino groups as discussed earlier to explain a limited cross-linking of glycophorins on intact cells (Marchesi & Furthmayr, 1976). Control experiments further demonstrated that the DTSP concentration remained sufficient (60–16% of control) to cross-link the majority of glycoproteins.

If preexisting protein–protein interactions were present on intact cells, amino group supplementation of the proteins should have increased exoplasmic cross-linking to a level observed with vesicles. Since the small extent of glycoprotein cross-linking on intact cells was observed under the same conditions that resulted in extensive cross-linking of similarly treated vesicles and isolated membranes, it reveals structural properties of the membrane. The data (i) indicate that intact erythrocytes have a restricted lateral mobility for these glycoproteins and (ii) suggest a monomeric arrangement for band 3 protein. Indirect evidence earlier suggested that band 3 forms dimers in erythrocyte membranes (Nigg & Cherry, 1979) and cross-linking data from isolated membranes pointed in the same direction (Wang & Richards, 1974; Clarke, 1975; Yu & Steck, 1975; Kiehm & Ji, 1977). The discrepancy between band 3 cross-linkability on intact cells and on isolated membranes must not necessarily imply that band 3 exists as a monomer. Conformational arrangements of expolasmic portions of associated monomers, primarily carbohydrates, could prevent cross-linking. However, amino group supplementation of oligosaccharide side chains that overcomes a possible lack of amino groups should decrease such effects of steric hindrance. Since it does not increase cross-linking substantially, a monomeric arrangement of band 3 is more likely. Staros et al. recently presented evidence for a successful cross-linking of band 3 protein on intact cells by an impermeable cross-linker (Staros et al., 1981). However, the reagent was applied under conditions that must result in cross-links by diffusion-controlled collisions and therefore do not allow to monitor preexisting associations (11.8 mM of reagent for 30 min at room temperature). The need for an impermeable, cleavable cross-linker is evident, but it should, at the same time, be very reactive to allow short incubations at low temperature at which lateral diffusion is largely reduced. We used the reactive cross-linker DTSP (Lomant & Fairbanks, 1976) and applied it at 0–4 °C for up to 8 min at low concentrations to prevent excess monofunctional reactions and to minimize indirect cross-linking by penetrating reagent. DTSP concentrations up to 1 mM did not increase the extent of cross-linked material on intact erythrocytes. For concentrations exceeding 1 mM, DTBP was applied for solubility reasons. However, no substantial increase in cross-linked labeled protein was observed (not shown), but slightly increased amounts of cross-linked spectrin and hemoglobin were detected but clearly not to the extent observed at elevated temperatures (Wang & Richards, 1975).

In cross-linking experiments with glycophorin-modified samples, we studied exclusively the formation of complexes larger than PAS_1. Thus, possible complexes involving the minor glycophorins which comprise 18% of all sialoglycoproteins (Owens et al., 1980) remained undetected as long as they did not reach apparent molecular weights exceeding that of PAS_1. Glycophorin A with 15 oligosaccharide side chains, each bearing two terminal sialic acid groups [for a review, see Tanner (1978)], was heavily modified by our labeling technique; nevertheless, cross-linkability of amino group supplemented glycophorins on intact cells was only 2 times higher than in the absence of additional aminogroups. In fact, amino group supplementation resulted in a similar enhancement in cross-links for glycophorin-modified and band 3 modified cells. This concerted behavior may indicate a close association of glycophorin with band 3 as suggested earlier (Wang & Richards, 1974; Clarke, 1975; Yu & Steck, 1975; Kiehm & Ji, 1977; Nigg & Cherry, 1979; Staros et al., 1981). Since no evidence was found for band 3–glycophorin interactions, the presumptive association probably occurs on the cytoplasmic side via cytoskeletal elements that retain the two proteins physically well separated on the exoplasmic side. Such associations no longer exist in spectrin-free vesicles and their absence may explain why the two glycoproteins undergo cross-linking independently, resulting in a 1.7-fold higher cross-linking for glycophorin than for band 3. This difference is probably due to the high degree of amino group supplementation of glycophorins. In contrast to this, cross-linkability of glycophorins is meager on isolated membranes and remains unchanged by amino group supplementation. This and the greatly enhanced cross-linkability of band 3 on isolated membranes indicate that protein–protein interactions in the membrane change dramatically during cell lysis. This fact has not been demonstrated by previous cross-linking studies [for a review, see Marchesi & Furthmayr (1976)], though it has earlier been recognized by an alteration in the arrangement of bound Con A molecules and intramembraneous particles, being well separated from one another and almost regularly

distributed on intact cells but irregularly clustered on isolated membranes (Bächi & Schnebli, 1975; Kuettner & Staehelin, 1976).

This cross-linking study on intact erythrocytes aided by amino group supplementation reveals that glycoproteins are maintained in a spatial arrangement with little lateral interactions that only slightly increase with cell age. This cell age dependent difference in cross-linkability is apparently minute but may involve a few hundred or at the most a few thousand molecules of band 3 per cell. It is likely that cross-linkable dimers or oligomers of a subpopulation of band 3 protein play a physiological role by facilitating IgG autoantibody binding to senescent cells as shown elsewhere (Lutz, 1981; H. U. Lutz and G. Stringaro, unpublished results).

The lack of protein–protein interactions on the surface of intact cells is consistent with stringent cytoskeletal restraints imposed on integral membrane proteins. The data suggest two types of restrictions, one that is affected by ATP depletion which results in detachment of cytoskeletal elements from the membrane and gives rise to spectrin-free vesicles in which glycophorins and band 3 readily undergo cross-linking. The second type of restriction is destroyed by a low ionic strength treatment. Absence of this restriction results in extensive band 3 cross-linking but barely affects the restriction imposed on glycophorins. Such restrictions should be detectable as immobilizations of glycoproteins when analyzed by either rotational (Nigg & Cherry, 1980) or lateral mobility measurements (Peters, 1981). However, these measurements have not been carried out on *intact* erythrocytes because of technical problems (Peters, 1981). Whether determined on isolated membranes (ghosts) (Nigg & Cherry, 1979, 1980; Golan & Veatch, 1980) or on fused erythrocytes (Fowler & Branton, 1977; Schindler et al., 1980), some type of restriction was destroyed. Thus, we do not know whether the fractional immobilization of band 3 measured by either technique is considerably higher on intact cells than on isolated membranes. Until this question can be answered, our data on a monomeric arrangement of band 3 remain suggestive evidence.

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References

Bächi, T., & Schnebli, H. P. (1975) *Exp. Cell Res. 91*, 285–296.

Bächi, T., Dorval, G., Wigzell, H., & Binz, H. (1977) *Scand. J. Immunol. 6*, 241–246.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Capaldi, R. A. (1973) *Biochem. Biophys. Res. Commun. 50*, 656–661.

Childs, R. A., Feizi, T., & Tonegawa, Y. (1979) *Biochem. J. 181*, 533–538.

Clarke, S. (1975) *J. Biol. Chem. 250*, 5459–5469.

Dejter-Juszynski, M., Harpaz, N., Flowers, H. M., & Sharon, N. (1978) *Eur. J. Biochem. 83*, 363–373.

Dottavio-Martin, D., & Ravel, J. M. (1978) *Anal. Biochem. 87*, 562–565.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fehr, J., & Knob, M. (1979) *Blood 53*, 966–976.

Fowler, V., & Branton, D. (1977) *Nature (London) 268*, 23–26.

Gahmberg, C. G. (1976) *J. Biol. Chem. 251*, 510–515.

Gahmberg, C. G., & Andersson, L. C. (1977) *J. Biol. Chem. 252*, 5888–5894.

Golan, D. E., & Veatch, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2537–2541.

Jeffrey, A. M., Zopf, D. A., & Ginsburg, V. (1975) *Biochem. Biophys. Res. Commun. 62*, 608–613.

Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.

Jentoft, N., & Dearborn, D. G. (1980) *Anal. Biochem. 106*, 186–190.

Ji, T. H. (1973) *Biochem. Biophys. Res. Commun. 53*, 508–514.

Ji, T. H. (1979) *Biochim. Biophys. Acta 559*, 39–69.

Kiehm, D. J., & Ji, T. H. (1977) *J. Biol. Chem. 252*, 8524–8531.

Koscielak, J., Zdebska, E., Wilczynska, Z., Miller-Podraza, H., & Dzierzkowa-Borodej, W. (1979) *Eur. J. Biochem. 96*, 331–337.

Krusuis, T., Finne, J., & Rauvala, H. (1978) *Eur. J. Biochem. 92*, 289–300.

Kuettner, C. A., & Staehelin, L. A. (1976) *Biochim. Biophys. Acta 448*, 114–120.

Lane, C. F. (1975) *Synthesis*, 135–145.

Liao, T. H., Gallop, P. M., & Blumenfeld, O. O. (1973) *J. Biol. Chem. 248*, 8247–8253.

Liu, S. C., Fairbanks, G., & Palek, J. (1977) *Biochemistry 16*, 4066–4074.

Lomant, A. J., & Fairbanks, G. (1976) *J. Mol. Biol. 104*, 243–261.

Lutz, H. U. (1978) *J. Supramol. Struct. 8*, 375–389.

Lutz, H. U. (1981) *Schweiz. Med. Wochenschr. 111*, 1507–1517.

Lutz, H. U., & Fehr, J. (1979) *J. Biol. Chem. 254*, 11177–11180.

Lutz, H. U., Lomant, A. J., McMillan, P., & Wehrli, E. (1977a) *J. Cell Biol. 74*, 389–398.

Lutz, H. U., Liu, S. C., & Palek, J. (1977b) *J. Cell Biol. 73*, 548–560.

Lutz, H. U., Däniken, A. V., Semenza, G., & Bächi, T. (1979) *Biochim. Biophys. Acta 552*, 262–280.

Marchesi, V. T., & Furthmayr, H. (1976) *Annu. Rev. Biochem. 45*, 667–698.

Müller, H., Schmidt, U., & Lutz, H. U. (1981) *Biochim. Biophys. Acta 619*, 462–470.

Neville, D. (1971) *J. Biol. Chem. 246*, 6328–6334.

Nigg, E. A., & Cherry, R. J. (1979) *Nature (London) 277*, 493–494.

Nigg, E. A., & Cherry, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4702–4706.

Orr, G. A. (1981) *J. Biol. Chem. 256*, 761–766.

Owens, J. W., Mueller, T. J., & Morrison, M. (1980) *Arch. Biochem. Biophys. 204*, 247–254.

Peters, R. (1981) *Cell Biol. Int. Rep. 5*, 733–760.

Rando, R. R., Orr, G. A., & Bangerter, F. W. (1979) *J. Biol. Chem. 254*, 8318–8323.

Schindler, M., Koppel, D. E., & Sheetz, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1457–1461.

Smith, D. F., & Ginsburg, V. (1980) *J. Biol. Chem. 255*, 55–59.

Staros, J. V., Haley, B. E., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 5004–5007.

Staros, J. V., Morgan, D. G., & Appling, D. R. (1981) *J. Biol. Chem. 256*, 5890–5893.

Steck, T. L. (1972) *J. Mol. Biol. 66*, 295–305.

Steck, T. L. (1974) *J. Cell Biol. 62*, 1-19.
Tanner, M. J. A. (1978) *Curr. Top. Membr. Transp. 11*, 279-325.
Tuech, J. K., & Morrison, M. (1974) *Biochem. Biophys. Res. Commun. 59*, 352-360.
Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005-8018.
Wang, K., & Richards, F. M. (1975) *J. Biol. Chem. 250*, 6622-6626.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9176-9184.

Changes in Membrane Potential of *Escherichia coli* in Response to Temporal Gradients of Chemicals†

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ABSTRACT: Changes in membrane potential of *Escherichia coli* in response to addition of chemoattractants have been studied by several groups, but their observations and conclusions disagree [e.g., Szmelcman and Adler [Szmelcman, S., & Adler, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4387-4391] and Snyder et al. [Snyder, M. A., Stock, J. B., & Koshland, D. E., Jr. (1981) *J. Mol. Biol. 149*, 241-257]]. This study was undertaken to resolve the differences in these reports. The discrepancies were probably the consequence of differences in the energy level of the bacteria, caused by differences in the availability of oxygen. In the presence of oxygen the relatively small changes in membrane potential that may be correlated with chemotaxis could be masked and compensated for by the changes in membrane potential caused by respiration and the related electrogenic transport processes. In the present study the contribution of the respiratory and related systems was reduced by using electron transport inhibitors such as KCN or amytal or 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO). Membrane potential was then monitored by tetraphenylphosphonium distribution in response to the addition of a chemoeffector stimulus. Addition of the chemoattractant galactose caused an increase in the membrane potential only if respiration was inhibited. This hyperpolarization was not caused by ATP hydrolysis since *N*,*N*'-dicyclohexylcarbodiimide (DCCD), an ATPase inhibitor, did not prevent it but rather increased it. The inhibitors used did not abolish the motility or the chemotactic response of the bacteria. Nine other attractants, metabolizable and nonmetabolizable chemicals, were tested under these conditions. All caused hyperpolarization, independent of the receptor identity or the chemotaxis focusing system with which they interact. The significance of these results and of earlier works in light of the present observations is discussed. This study neither proves nor disproves the possible correlation between the observed hyperpolarization and the process of chemotaxis. It describes the conditions that enable the detection of these changes in membrane potential.

Motile bacteria swim toward certain chemicals (attractants) and away from others (repellents), a phenomenon called chemotaxis. In the absence of a stimulating chemical, the motion of bacteria such as *Escherichia coli* is composed of runs in a straight line interrupted by brief tumbles. In an increasing gradient of attractant or a decreasing gradient of repellent, the cells tumble less frequently, with a resultant aggregation toward the attractant or away from the repellent [for recent reviews, see Adler et al. (1979), Koshland (1980a), and Macnab (1980)]. The machinery of bacterial chemotaxis can, in principle, be thought of as the bacterial version of a nervous system. In analogy to eucaryotic nerve cells and protozoa where sensory transduction is mediated by an action potential (Aidley, 1971; Kung et al., 1975), studies have been carried out to determine if membrane potential is involved in the signaling process of bacterial chemotaxis. These studies were of two types: (a) studies of the behavior of bacteria at different levels of membrane potential and (b) measurements of changes in membrane potential in response to addition of chemoattractants.

The investigations in the first category yielded conclusive results: the level of membrane potential affected the behavior of bacteria (de Jong et al., 1976; Manson et al., 1977; Miller & Koshland, 1977, 1980; Khan & Macnab, 1980; Goulbourne & Greenberg, 1981) and consequently a distinct membrane potential signaling protein was postulated (Laszlo & Taylor, 1981).

For the investigations in the second category, the results were in apparent disagreement (Szmelcman & Adler, 1976; Miller & Koshland, 1977; Armitage & Evans, 1979, 1981; Snyder et al., 1981). Even in a single species, *E. coli*, conflicting results were obtained: Szmelcman & Adler (1976) detected changes in membrane potential in response to attractants, but Snyder et al. (1981) and Armitage & Evans (1981) did not find such changes. Snyder et al. reported that a few attractants did cause changes in membrane potential, but these changes were not related to chemotactic sensing. On the other hand, in all of these studies, it was found that some repellents, such as acetate, lead to permanent hyperpolarization[1] in *E. coli* (Szmelcman & Adler, 1976; Snyder et al.,

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