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Selective Labeling of the Hydrophobic Core of Membranes with 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine, a Carbene-Generating Reagent†

Josef Brunner and Giorgio Semenza*

ABSTRACT: The synthesis of a new photoactivatable probe, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID), with a high specific radioactivity (10 Ci mmol^{-1}) is described. It was tested as a probe for the hydrophobic core of membranes. TID partitions strongly in favor of the lipid phase of membranes, and the photogenerated carbene labels intrinsic membrane proteins in a highly selective manner. This conclusion was reached from the distribution of radioactivity among the proteins of [^{125}I]TID-labeled human erythrocyte membranes. By far the most heavily labeled protein is band 3 [nomenclature of Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617] while the labeling of glycophorin is approximately 5 times less than that of band 3. There is little or no labeling of known extrinsic proteins.

The principal goal of hydrophobic membrane labeling is the identification of those domains of proteins that penetrate the lipid core. When those polypeptide segments which are in contact with the fatty acyl chains of the membrane lipids are selectively modified, this method represents a valuable complement to existing surface labeling techniques.

Most of the reagents which are in current use for hydrophobic membrane labeling are photoactivatable precursors of aryl nitrenes (Klip & Gitler, 1974; Bercovici & Gitler, 1978; Kahane & Gitler, 1978; Wells & Findlay, 1979a,b; Cerletti & Schatz, 1979) or of carbenes (Bayley & Knowles, 1978b; Gupta et al., 1979; Brunner & Richards, 1980). These precursors are hydrophobic or amphipathic and partition to a high extent into the lipid core of membranes. Unlike classical protein modifying reagents, nitrenes and carbenes are very reactive and exhibit less chemical selectivity. In model membrane systems prepared from saturated phospholipids, carbenes have been shown to react to some extent even with aliphatic residues. Therefore, carbenes are particularly attractive candidates for the chemical modification of the hydrophobic and chemically predominantly inert polypeptide side chains. Aryl nitrenes are less reactive than corresponding carbenes, and C–H insertion does not substantially, if at all, contribute to the overall labeling process (Bayley & Knowles, 1978a; Gupta et al., 1979; Brunner & Richards, 1980).

Nitrenes and carbenes are susceptible to intramolecular rearrangements which result in the transient appearance of second intermediates which not only are less reactive than nitrenes and carbenes but also may be more polar than the precursors. Since these species are assumed to be relatively long-lived (as compared to nitrenes and carbenes), the probability is increased that reactive amino acid residues are modified that are located on the surface area of proteins which faces the aqueous medium. That labeling of proteins may be dominated by group-specific reactions involving such intermediates has been proposed recently (Hu & Wisnieski, 1979).

[^{3}H]Adamantanyldiazirine generates a highly reactive carbene and labels intrinsic membrane proteins far more heavily than the extrinsic proteins (Goldman et al., 1979; Bayley & Knowles, 1980). As suggested by these investigators, the reaction of extrinsic proteins may well be reduced or even eliminated if reagents more hydrophobic than adamantanyldiazirine were available.

When [^{3}H]adamantanyldiazirine in native membranes is used the labeling of various proteins was found to occur predominantly in those polypeptide segments which presumably span the hydrophobic layer. In the case of glycophorin, however, the extent of labeling was reduced by the presence of glutathione in the water phase (Bayley & Knowles, 1980). This showed that some reaction did take place at sites which were accessible to the water-soluble thiol. As the authors suggested, these sites exposed to water might have reacted not with the carbene but with another intermediate (for example,

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with diazoadamantane which is known to be produced in high yield from the diazirine by photoisomerization).

On the basis of a previous study on 3-(trifluoromethyl)-3-phenyldiazirine (Brunner et al., 1980), we have now synthesized and examined the new carbene generating reagent 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID).[1] This reagent can be easily prepared with very high specific radioactivity. We demonstrate that TID combines a number of properties which highly recommend this compound as a hydrophobic labeling reagent.

Experimental Procedures

(*1*) *Chemical Syntheses.* All chemicals and solvent were commercial grades of highest purity. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates from Merck. ^1H NMR spectra (100 MHz) were measured on a Varian HA-100 using $CDCl_3$ as a solvent and $(CH_3)_4Si$ (δ = 0.00) as an internal standard. Chemical shifts are given in parts per million. Infrared (IR) spectra were recorded on a Perkin-Elmer 257 spectrometer.

m-Iodotrifluoroacetophenone Oxime (*III*). Starting material was trifluoroacetophenone (Fluka). *m*-Aminotrifluoroacetophenone (I) was obtained by procedures previously described (Stewart & Van der Linden, 1960; Klabunde & Burton, 1970). Diazotization of I followed by treatment with iodide gave *m*-iodotrifluoroacetophenone (II) (Klabunde et al., 1970). Ketone II (2.96 g, 10 mmol) and hydroxylamine hydrochloride (11 mmol) were refluxed in 10 mL of ethanol for 3 h while the apparent pH was maintained at ~5 by successive additions of 4 M NaOH. Precipitated NaCl was filtered and washed with ethanol. The combined filtrates were concentrated, and the residue was dissolved in ether and extracted twice with water. Evaporation of the ether yielded the oxime III: 3.10 g (98%); TLC (hexane/ether, 2:1 v/v) R_f 0.6; mp (crystallized from hexane) 85–87 °C. Anal. Calcd for C_8H_5INO: C, 30.50; H, 2.00; N, 4.45; I, 40.28. Found: C, 30.95; H, 1.70; N, 4.44; I, 39.71.

m-Iodotrifluoroacetophenone O-(p-Toluenesulfonyl)oxime (*IV*). Oxime III (2.73 g) and *p*-toluenesulfonyl chloride (1.74 g) were refluxed in 10 mL of pyridine for 3 h. The reaction mixture was concentrated and the residue dissolved in ether. The organic solution was extracted with 1 M HCl, washed with water, and then dried over $MgSO_4$. Evaporation of the solvent gave 3.39 g (83.5%) of the crude tosylate of which 470 mg was purified by TLC (hexane/ether, 2:1 v/v): R_f 0.5; mp 72–73 °C; ^1H NMR ($CDCl_3$) δ 2.44 (s, 3 H) and 7.2–7.9 (m, 8 H). Anal. Calcd for $C_{15}H_{11}F_3INO_3S$: C, 38.39; H, 2.36; N, 2.98; S, 6.83. Found: C, 38.71; H, 2.47; N, 2.91; S, 7.26.

3-(Trifluoromethyl)-3-(m-iodophenyl)diaziridine (*V*). In a hydrolysis tube (Pierce), tosylate IV (228 mg) was dissolved in ether (2 mL). This solution was cooled to −78 °C, and approximately 0.5 mL of liquid ammonia was added. The sealed tube was kept at 25 °C for 24 h. Then ammonia was carefully evaporated and the salty residue extracted with ether. The ether was extracted with water, dried over $MgSO_4$, and evaporated. The residual oil was dried at 10^{-2} torr for 2 h: yield 137 mg (90%); TLC (hexane/methylene chloride, 1:2 v/v), single spot (iodine), R_f 0.35 (tosylate R_f 0.60); ^1H NMR ($CDCl_3$) δ 2.2 and 2.8 (AB system, J = 10 Hz, 2 H) and 7–8 (m, 4 H).

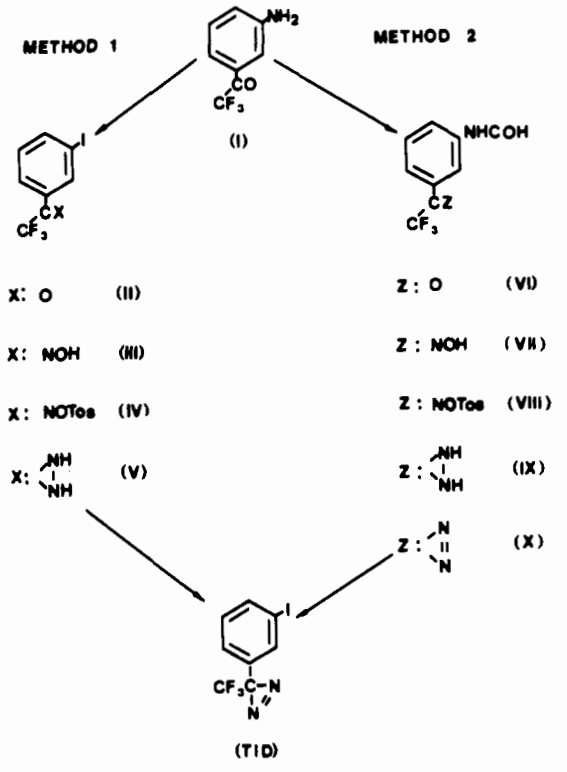

FIGURE 1: 3-(Trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID). Reaction scheme for the synthesis of TID by method 1 and method 2.

3-(Trifluoromethyl)-3-(m-iodophenyl)diazirine (*TID*). Diaziridine V was oxidized as described previously for the noniodinated analogue (Brunner et al., 1980) with silver oxide. Progress of the reaction was followed by measuring the absorption of the diazirine band at 350 nm. The reaction was quantitative (>98%). Silver oxide was removed by filtration and extracted with ether. Careful evaporation of the solvent at 50 °C yielded a slightly yellow residue which contained some residual ether: TLC (hexane) R_f 0.70. For UV spectrum, see Results.

3-(Trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]*TID*). By use of the procedure outlined above, radioactively labeled TID has been prepared of a specific radioactivity of approximately 1 Ci mmol^{-1}. All intermediates of the synthesis have been identified by cochromatography with samples of the nonlabeled compounds. Purifications were accomplished by TLC. The yield was approximately 5% (250 μCi) on the basis of $Na^{125}I$. The material was stored as a dilute solution in ether at −20 °C.

As depicted in Figure 1, an alternative method has been developed for the synthesis of TID. This synthesis (method 2) is described below.

m-(Formylamino)trifluoroacetophenone (*VI*). *m*-Aminotrifluoroacetophenone (12.9 g) and formic acid (99%) (Fluka) (30 mL) were heated at 100 °C for 1 h. Excess formic acid was removed by distillation, the oily residue was dissolved in 50 mL of benzene, and the solvent was evaporated again. The product was then crystallized from benzene (50 mL). TLC (chloroform/methanol, 8:1 v/v, one spot, R_f 0.60; yield 8.3 g (56%); mp 105–107 °C. Anal. Calcd for $C_9H_6F_3NO_2$: C, 49.87; H, 2.79; N, 6.14. Found: C, 48.97; H, 2.43; N, 6.32.

m-(Formylamino)trifluoroacetophenone Oxime (*VII*). Ketone VI (2.17 g, 10 mmol), hydroxylamine hydrochloride (20 mmol), and ethanol (20 mL) were refluxed for 2 h. The

[1] Abbreviations used: TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)-diazirine; $NaDodSO_4$, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; UV, ultraviolet; mp, melting point; PC, phosphatidylcholine; tosyl, *p*-tolylsulfonyl; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; LIS, lithium iodosalicylate.

HCl produced was neutralized by successive addition of 3 mL of 4 M NaOH. Throughout the reaction, the apparent pH was around 5. The solvent was evaporated, the residue dissolved in ether, and the inorganic salt extracted with water. The organic phase was evaporated and the residue (2.21 g) chromatographed on TLC plates (chloroform/methanol, 8:1 v/v). The oxime had an R_f value of 0.50. The material eluted from TLC plates spontaneously crystallized upon evaporation of the solvent (ether): yield ~30%; mp 148–149 °C. Anal. Calcd for $C_9H_7F_3N_2O_2$: C, 46.56; H, 3.04; N, 12.07. Found: C, 46.41; H, 3.14; N, 11.95.

m-(Formylamino)trifluoroacetophenone O-(p-Toluenesulfonyl)oxime (VIII). Oxime VII (241 mg, 1.04 mmol), 2.5 mL of absolute pyridine, and 1.20 mmol of *p*-toluenesulfonyl chloride were refluxed for 2 h. The solvent was evaporated, and the dark residue was dissolved in chloroform. TLC (ether) gave 130 mg of the pure tosylate as a slightly yellowish oil: ^{1}H NMR ($CDCl_3$) δ 2.51 (s, 3 H), 6.8–7.8 (m, 8 H), 8.15 (s, 1 H), and 8.55 (broad s, 1 H).

3-(Trifluoromethyl)-3-[m-(formylamino)phenyl]diazirine (X). Treatment of an etheral solution of 130 mg of the tosylate VIII with ammonia as described above for V gave the diaziridine IX. On TLC plates, the diaziridine had a slightly smaller R_f value (solvent/ether) than the tosylate. The diaziridine gives a bright yellow spot on exposure to iodine vapor and can easily be distinguished from educt. The product was purified by TLC (ether), and after the material was eluted from the silica gel with the same solvent, the solution (10 mL) was treated with 1 g of freshly precipitated silver oxide. Progress of the diazirine formation was monitored by UV spectroscopy of aliquots of the supernatant and by TLC (R_f values: diaziridine IX, 0.35; diazirine X, 0.55). The reaction was completed within 3 h [the time required for quantitative (98%) conversion may, however, be variable, depending on the batch of silver oxide used]. The oxide was filtered and extracted with ether, and the combined filtrates were concentrated. The residue crystallized from benzene/ether (1:1): yield 17 mg; mp 69–70 °C; UV max (methanol) 356 nm (ϵ_{356} 288) (diazirine). The product gave a single spot on the TLC (ether).

3-(Trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine (TID). Diazirine X (12.4 mg) was deformylated in a mixture of 120 μL of methanol and 60 μL of 12 M HCl (26 °C, 30 min). The reaction mixture was dried by means of a stream of nitrogen and the crystalline residue dissolved in 200 μL of methanol/water (1:1 v/v). A fraction of this stock solution (50 μL) was transferred into a small test tube, then the solvent was reevaporated, and the residue was dissolved in 100 μL of 3 M H_2SO_4. The aryl amine (not further characterized) was diazotized by successively adding at 0 °C 1–2-μL portions of 1 M $NaNO_2$ until a lasting (>30 min) positive reaction on KI–starch indicator stripes was observed (total 11 μL).

In a conical vial (0.3-mL Reactivial, Pierce) equipped with a Mininert valve (Pierce), 5 mCi of Na^{125}I (EIR, Würenlingen) was diluted with cold NaI to give 15 μL of a 50 mM solution of Na^{125}I (10 Ci/mmol). Diazonium salt (15 μL) was added to the ice-cold iodide, and the mixture (which immediately after mixing of the components turned turbid) was kept at 50 °C for 30 min. Trace amounts of I_2 were reduced by $NaHSO_3$ (50 μL of a 5% solution), and the lipophilic reaction products were extracted into 5 μL of CCl_4. During a centrifugation (2 min, 1000 rpm, MSE table centrifuge), the organic phase was collected in the tip of the conival vial and was easily transferred onto a TLC plate. The chromatogram was developed in hexane. TID was identified by its R_f value (0.7) (identical with that of TID prepared according to method 1) and precisely localized on the plate by fluorescence quenching (254 nm). The silica gel containing the radioactive product was scraped off and extracted with approximately 300 μL of ethanol, and the TID solution was stored in the dark at −20 °C. The yield based on introduced ^{125}I varied from 60% to 80%. The diazirine prepared by this method had a UV spectrum identical with that of TID prepared by method 1.

(*2*) *Photolabeling Experiments.* Human erythrocyte membranes were prepared according to the method of Steck & Kant (1974). Membranes were suspended in 5 mM sodium phosphate and 1 mM EDTA, pH 8, and stored at 4 °C for no longer than 3 days. For photolabeling, ghosts were deoxygenated by flushing with a gentle stream of nitrogen for at least 30 min at 0 °C. An appropriate volume of the [^{125}I]TID stock solution in ethanol was added (final concentration of ethanol <1%), and the mixture was equilibrated for 15 min at 0 °C. The membranes were transferred into a quartz cuvette equipped with a Teflon magnetic stirring bar and photolyzed. A 350-W medium pressure mercury lamp was used as a light source (Illumination Industries, Inc., Type 350-1008). The beam was directed through filters of circulating cold water (30 mm) and a saturated solution of copper sulfate (20 mm) [the latter efficiently screens out radiation shorter than 315 nm (Katzenellenbogen et al., 1974)] onto the center of the cuvette.

(*3*) *Analytical Procedure.* Protein was determined according to a modified Lowry procedure (Peterson, 1977) by using bovine serum albumin as a standard. Phospholipid was determined by the method of Chen et al. (1956). For Na$DodSO_4$–polyacrylamide gel electrophoresis, protein samples were denatured in 1% Na$DodSO_4$ and 10 mM DTT (30 min, 37 °C). Proteins were separated on a discontinuous electrophoresis system as described previously (Brunner et al., 1979).

(*4*) *Glycophorin: Purification and Trypsin Digestion.* Glycophorin was isolated from [^{125}I]TID-labeled human red cell membranes by the LIS–phenol method of Marchesi & Andrews (1971). The protein was then dissolved in 2% Na$DodSO_4$ and Tris–H_2SO_4 (25 mM), pH 8.0, and gel filtrated on Sepharose 6B with the same buffer (0.1% Na$DodSO_4$). This gel filtration was done to eliminate the possibility that noncovalently bound radiolabel remained associated with the glycophorin (Wells & Findlay, 1979a,b). Peak fractions containing glycophorin were pooled and lyophilized. The glycoprotein (1.3 mg) containing large amounts of Na$DodSO_4$ was dissolved in 1 mL of 6 M urea and Tris–acetate, pH 7.8, and the solution was passed through a column (1 × 5 cm) of Dowex 1-X2 which had been pretreated as described by Weber & Kuter (1971). Peak fractions containing radioactivity (recovery 60%) were pooled and dialyzed against distilled water. The glycoprotein was lyophilized and dissolved in 50 mM Tris-HCl, pH 8.5, at a concentration of 3 mg mL^{-1} (48 000 cpm/mg of protein) and centrifuged in an Eppendorf centrifuge (Model 5412) for 20 min. Of the supernatant, 100 μL (300 μg of protein) was transferred into an Eppendorf tube and mixed with 10 μg of trypsin (DPCC-trated, Type XI, Sigma). Digestion was performed at 37 °C for 12 h. The trypsin was inactivated by the addition of (N^{α}-tosyl-L-lysyl)-chloromethane (2 μg) as described earlier (Furthmayr et al., 1978), and the solution was acidified with acetic acid (pH 4). The precipitate was pelleted (2 min; Eppendorf), redissolved at pH 7.5, and precipitated again.

Results

Synthesis and Stability of 3-(Trifluoromethyl)-3-(m-

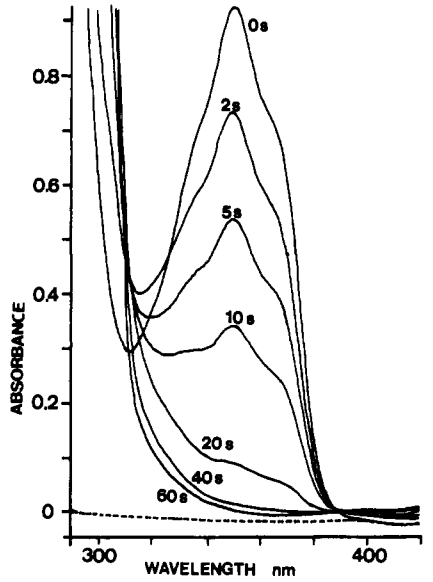

FIGURE 2: Time course for the photolytic decay of 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine. A solution of TID, ~3 mM, in ethanol was photolyzed for different length of time (0, 2, 5, 10, 20, 40, and 60 s), and the ultraviolet spectra were recorded with a Unicam SP 1700 spectrometer.

[^{125}I]iodophenyl)diazirine. The reagent was prepared by two routes as described under Experimental Procedures (Figure 1). Both pathways follow the reaction sequence that has been worked out previously for the synthesis of the noniodinated analogue, 3-(trifluoromethyl)-3-phenyldiazirine (Brunner et al., 1980). A central feature of method 2 is that the diazirine was formed in the presence of a masked amino group in the same molecule (Figure 1, VI–X). We have found that the formyl group is a suitable amine protective group during the synthesis of the diazirine and, in addition, that the diazirine is sufficiently stable in strong acid to allow subsequent removal of the formyl group and recovery of the aryl amine. Diazotization of the amine, substitution of the diazo group by iodine, is a standard reaction to introduce iodine into an aromatic system. We surmise that method 2 is likely to be the method of choice for the synthesis of radioactive TID because the radioiodine is introduced in the very last step of the synthesis which does not require extensive manipulation of radioactive material.

When stored in ethanol at −20 °C, [^{125}I]TID showed approximately 2.5% self-decomposition within two weeks (TLC, hexane) at a radiolabel concentration of 25–30 μCi μL^{-1} (10 Ci mmol^{-1}). As a dilute solution in ether (1 μCi μL^{-1}), the reagent is stable for at least 4 weeks. On irradiation with UV light, the diazirine is rapidly photolyzed as determined spectroscopically by the disappearance of the diazirine absorption band at 350 nm (Figure 2). Under the experimental conditions used, the half-time of the decay is approximately 7 s.

Hydrophobicity of the Reagent. In order to compare the hydrophobicity of TID with that of other reagents used for hydrophobic membrane labeling (Bercovici & Gitler, 1978; Wells & Findlay, 1979a,b, 1980; Bayley & Knowles, 1980), we determined the partitioning of TID between membranes and the aqueous buffer. The procedure we used has essentially been described by Bayley & Knowles (1980). Here, we define the partition coefficient P_L as P_L = (ligand bound/milligram of lipid)/(free ligand/microliter of external solution). The results are shown in Table I. When P_L was measured with egg PC liposomes or with small-intestinal brush-border membrane vesicles, a value was found for TID which is approximately 6–8 times lower than that of iodonaphthyl azide. When our data are compared with those obtained for iodonaphthyl azide and adamantanyldiazirine (Bayley & Knowles, 1980), it is evident that TID is more hydrophobic than adamantanyldiazirine.

Table I: Partition Coefficients of Hydrophobic Photolabeling Reagents between Membranes and Aqueous Buffer at 25 °C[a]

reagent	single bilayer egg PC liposomes[b]	erythrocyte membranes	brush-border[d] membranes
TID	4.1×10^4 ($\pm 0.5 \times 10^4$ SD; $n = 2$)	3.9×10^4 [c]	6.5×10^4 [f]
5-iodonaphthyl 1-azide[e]	2.8×10^5 ($\pm 0.3 \times 10^5$ SD; $n = 3$)	ND[h]	3.6×10^5 [g]

[a] The partition coefficient P_L is defined as P_L = (ligand bound/milligram of lipid)/(free ligand/microliter external solution). [b] Egg PC, 3.5 mg/mL; reagent, ~1 μM. [c] Erythrocyte membranes, 0.7 mg of lipid/mL; reagent, ~1 μM. [d] Brush-border membranes were prepared as described previously (Kessler et al., 1978). [e] This reagent was prepared according to the procedure described by Cerletti & Schatz (1979); the specific radioactivity was 1 Ci/mmol. [f] Reagent concentration, 0.5–200 μM; lipid, 0.7 mg/mL. [g] Reagent concentration, ~5 μM; lipid, 0.7 mg/mL. [h] ND, not determined.

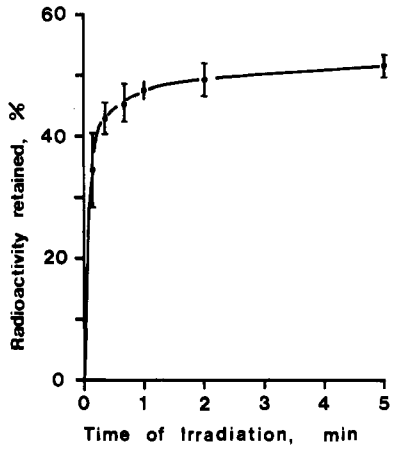

FIGURE 3: Time course of the photochemical labeling of erythrocyte ghosts with [^{125}I] 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine. The curve represents the percentage of radioactivity originally present in the mixture that could not be removed from the membranes as a function of time of exposure to light. Erythrocyte membranes (2.18 mg of protein/mL; 5 mM sodium phosphate, pH 7.6) were incubated with [^{52}I]TID (2 μM; 10 Ci mmol^{-1}) and irradiated. At each time point, 50-μL aliquots of the membrane dispersion were diluted 40-fold with sodium phosphate buffer (5 mM; pH 8) containing 1% bovine serum albumin, and the membranes were recovered by centrifugation (30 min; 15000*g*). Pelleted ghosts were washed another 3 times with the same buffer and twice more with albumin-free buffer. Each point represents the mean value of three photolysis experiments (±SD).

The partition coefficient was determined at different ligand concentrations. Within a range from 0.5 to 200 μM, there was no detectable difference. If it is assumed that the bound ligand is dissolved in the lipid phase of the membrane, then the actual concentration of the reagent in the membrane lipid can be estimated. In the experiments reported in Table I where as much as 200 nmol of ligand was dissolved in 0.7 mg of lipid, the reagent concentration in the lipid phase approached a value of approximately 0.5 M.

Labeling of Erythrocyte Ghosts. When [^{125}I]TID equilibrated with erythrocyte membranes was irradiated, the ghosts covalently incorporated radiolabel which could not be removed with buffer containing bovine serum albumin. Incorporation of label was very rapid during the initial period of photolysis (Figure 3). After 1 min, a level was reached corresponding to 48% (±5%; SD; n = 4) of the total radioactivity that was

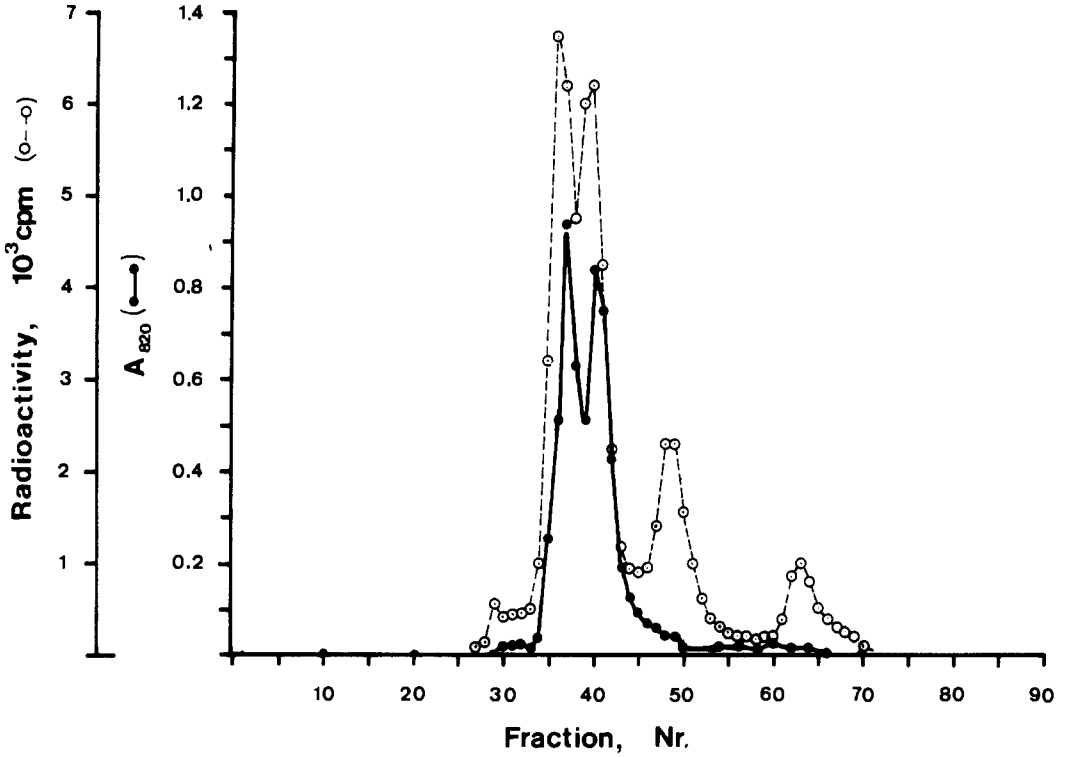

FIGURE 4: Elution profile of lipids extracted from [^{125}I]TID-labeled human erythrocyte membranes on Sephadex LH-20. Labeled membranes were washed 4 times with serum albumin and extracted with chloroform/methanol (2:1 v/v) following the general procedure of Folch et al. (1957). The organic phase was dried and the residue (15 mg) dissolved in methanol/chloroform (1:1 v/v). Extracted lipids were fractionated by gel filtration on Sephadex LH-20 (1.5 × 85 cm) using chloroform/methanol as the solvent. The flow rate of the column was controlled by a constant hydrostatic pressure (1.3 m). Fractions (2 min; 1.3 mL) were collected and aliquots used to determine radioactivity and phosphorus (Chen et al., 1956).

originally present. Prolonged photolysis for 5 min did not significantly alter this value. The time course of the incorporation of radioactivity is in good correlation with the decay of the diazirine, under identical conditions of irradiation. A photolysis time of 5–10 s is required to reach half of the plateau level. This half-time value is comparable with that of the disappearance of the diazirine in the experiment of Figure 2 (7 s).

For determination of the distribution of radiolabel among the components of the membrane, labeled ghosts were subjected to various analytical procedures. When separated by $NaDodSO_4$–polyacraylamide gel electrophoresis (6–8% polyacrylamide), 94.7% (mean of four experiments) of the radioactivity associated with the membrane ran with or ahead of the tracking-dye front. The radiolabel in this region is due to labeled lipid and photolysis products of [^{125}I]TID which had not been removed completely by bovine serum albumin. Lipid and protein of labeled ghosts were separated by a chloroform–methanol extraction. As approximately 85% of the radioactivity was recovered in the organic phase, this would, in addition to lipids, contain photolysis products of [^{125}I]TID if such were present to a substantial amount. The organic phase was then subjected to a gel filtration on Sephadex LH-20 using chloroform/methanol (1:1 v/v) as the solvent. Figure 4 reports the elution profiles of radioactivity and phosphorus (phospholipids). Essentially all of the phosphorus appeared within fractions 34–45 which in addition contained 65% of the radioactivity applied to the column. Since the shapes and positions of both elution profiles are almost identical, this strongly suggests that the radioactivity is bound to phospholipids. A second peak of radioactivity (fractions 46–53), corresponding to 24% of the radioactivity, is very likely due to labeled cholesterol. Indeed, on evaporation of the solvent of these fractions, white crystals were formed, and this material could be identified unequivocally as cholesterol. Finally 6% of the radioactivity was eluted with or closely to the total volume of the column. This material has not been characterized further, and it may represent some radioactivity which is not covalently bound to any component of the membrane. From this analysis, we conclude that at least 80% of the radiolabel that was not extracted by serum albumin from labeled ghosts must be due to labeled lipid (phospholipids and cholesterol). As only approximately 5% of the radioactivity is bound to proteins, the estimated lipid to protein labeling ratio is approximately 16 in [^{125}I]TID-labeled human erythrocyte membranes.

Band 3 protein and glycophorin A dimers (PAS I) can be separated completely by $NaDodSO_4$–gel electrophoresis on a polyacrylamide gel (8.4 × 2.7).[2] This is demonstrated in Figure 5 (PAS) which shows glycoproteins stained with fluorescent dansylhydrazin (Eckhardt et al., 1976). Afterwards, the gel was equilibrated with acetic acid (7%) and stained with Coomassie Brilliant Blue R-250 (Figure 5, CB). The protein pattern was essentially identical with those reported in the literature (e.g., Fairbanks et al., 1971). Then, the gel was dried and autoradiographed (Figure 5, A). Since it was of interest to detect a possible scavenging effect of glutathione on the intermediate carbene, labeling of ghosts was performed both in the presence and in the absence of this water-soluble thiol.

As demonstrated by the autoradiography, most of the label associated with protein was found in band 3. Additional radioactivity was present at positions that contain other known

[2] As suggested (Hjertén, 1962), the first figure in the parentheses indicates the total concentration of monomers (gram percent, w/v) and the second that of *N,N'*-methylenebis(acrylamide), as percentage of the total monomer concentration (w/w).

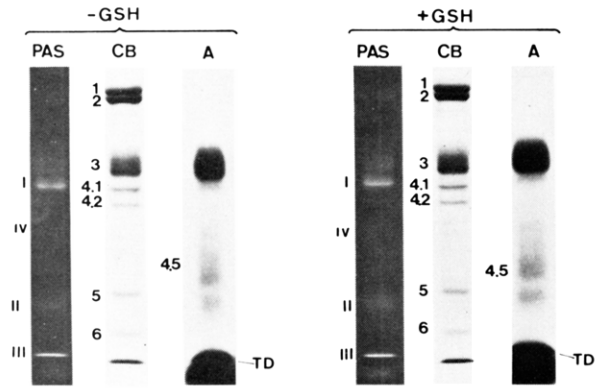

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of human erythrocyte membranes labeled with [^{125}I]TID in the absence (–GSH) and in the presence (+GSH) of reduced glutathione (50 mM). Red cell membranes (1.09 mg of protein/mL) were equilibrated with [^{125}I]TID (10 μM; 10 Ci mmol^{-1}) and irradiated for 60 s. The membranes were washed as described in the legend of Figure 3. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed on a slab gel (8.4 × 2.7);[2] samples of labeled membranes (30 μg of protein) were treated with $NaDodSO_4$ (1%) and DTT (10 mM) at 37 °C for 30 min prior to electrophoresis. PAS, periodic acid–Schiff (dansylhydrazin) stained glycoproteins (Eckhardt et al., 1976). The numerals (I–IV) indicate glycophorin according to the nomenclature of Fairbanks et al. (1971). CB, Coomassie-Brilliant Blue R-250 stained proteins (same nomenclature as above). A, Autoradiography of the dried slab gel (Kodak X-Omat; exposure time 28 h at –80 °C).

intrinsic proteins (PAS I–IV and the region of band 4.5). On the other hand, *none* of the extrinsic proteins (bands 1, 2, 4.1, 4.2, 5, and 6) was labeled significantly. Furthermore, as judged from the autoradiography, there seems to be no difference in the label distribution whether glutathione had been present during the photolysis or not.

For quantitation of the incorporation of label into individual proteins, protein bands were cut off, and the amount of label was determined by γ counting. These results are presented in Table II. This table confirms the lack of an effect of glutathione on the labeling efficiency and distribution of label among the proteins. This result is in contrast to what was observed by labeling ghosts with [^{3}H]adamantanyldiazirine (Bayley & Knowles, 1980). In their experiment, a significant reduction of the specific radioactivity of glycophorin was found when glutathione had been present during the labeling procedure. Furthermore, Table II demonstrates that the quantitative distribution of radioactivity among the proteins is quite different from that obtained with adamantanyldiazirine or from those found upon aryl azide labeling (Kahane & Gitler, 1978; Wells & Findlay, 1979a,b) (see Discussion).

Glycophorin was isolated from [^{125}I]TID-labeled ghosts by the LIS–phenol method of Marchesi & Andrews (1971). Final purification (removal of noncovalently bound radioactivity) was done by $NaDodSO_4$–polyacrylamide gel electrophoresis. The protein (PAS I) was extracted from the gel and its specific radioactivity determined. From this experiment it was concluded that 0.35% of the original radioactivity was associated with glycophorin (see footnote *e* of Table II). This value is in good correlation with the number calculated for PAS I protein (Table II). In fact, under the conditions of protein denaturation (30 min, 37 °C, 1% $NaDodSO_4$), PAS I protein represents a fraction only of the total glycophorin (Furthmayr & Marchesi, 1976).

In an attempt to determine the amount of radioactivity associated with spectrin (bands 1 and 2), appropriate correction had to be made due to some background radioactivity along the entire track of the gel (approximately 100 cpm per slice of 1 mm). It was estimated that the *maximal* fraction of label

Table II: Distribution of Label (Iodine-125) in the Components of the Erythrocyte Membrane

	percentage of radioactivity when labeled in the	
	absence of glutathione	presence of glutathione (50 mM)
total radioactivity in the photolysis sample[a]	100 (=3.7 × 10^8 cpm)	100 (=3.7 × 10^8 cpm)
membranes after extraction of non-bound label[b]	52	54
label associated with lipid[c]	≥40	≥40
label associated with protein	2.7	2.8
label in band 3	1.3 (±0.1 SD; n = 4)	1.2 (±0.1 SD; n = 4)
label in PAS I[d]	0.20 (±0.02 SD; n = 4)	0.20 (±0.02 SD; n = 4)
label bound to glycophorin when isolated by LIS–phenol[e]	0.35	ND[f]
label in spectrin (bands 1 and 2)	≤0.01	≤0.01

[a] Membranes (1.09 mg of protein/mL) were equilibrated with [^{125}I]TID (3.7 × 10^8 cpm mL^{-1}) and irradiated for 60 s. [b] Non-bound label was removed from the membranes by washing with buffer containing 1% bovine serum albumin. [c] Washed membranes were treated with 1% (w/v) $NaDodSO_4$ and 10 mM DTT (30 min, 37 °C) and subjected to $NaDodSO_4$-gel electrophoresis. Labeled lipid migrated ahead or with the tracking-dye front. [d] Glycoproteins were stained according to Eckhardt et al. (1976). [e] Glycophorin was isolated by the LIS-phenol method (Marchesi & Andrews (1971) from ghosts (246 mg of protein) which were supplemented with 50 μL of [^{125}I]TID-labeled ghosts (1.9 × 10^7 cpm). The sialoglycoprotein was further purified by $NaDodSO_4$-gel electrophoresis. The specific radioactivity of the isolated glycophorin was 0.91 × 10^4 cpm/mg of protein. The number is calculated on the basis of glycophorin being approximately 3% of the total protein in erythrocyte membranes. [f] ND, not determined.

which was bound to spectrin was 0.01% of the radioactivity originally present or approximately 0.2% of that fraction of radioactivity which was associated with protein.

Glycophorin Is Labeled within a Segment Spanning the Membrane. $NaDodSO_4$–polyacrylamide gel electrophoresis of glycophorin isolated from [^{125}I]TID-labeled red cell membranes and purified by gel filtration in a buffer containing $NaDodSO_4$ showed no major contaminants (Figure 6). The radioactivity profile shows four major peaks corresponding to positions which contain glycoprotein as detected by periodic acid–Schiff's reagent (PAS) (Fairbanks et al., 1971). A cylindrical gel stained with Coomassie Brilliant Blue R-250 (CB) revealed one possible contaminant migrating slightly ahead of glycophorin dimers (PAS I). Upon trypsin digestion of this preparation of glycoprotein and acidification, a dense precipitate was formed. This insoluble material corresponds to a peptide designated as T_{is} or T-6 (Furthmayr et al., 1978) and includes a sequence of uncharged amino acid residues presumed to be located within the lipid core of the membrane. The precipitate was pelleted, and the supernatant soluble peptides were decanted. Essentially all (96%) of the radioactivity was found in the insoluble material. This pellet could be solubilized by adjusting the pH to 7.5 and precipitated again by lowering the pH to 4. The peptide was collected by centrifugation and dissolved in a buffer containing 2% $NaDodSO_4$. The solution was boiled for 2 min, and the samples were

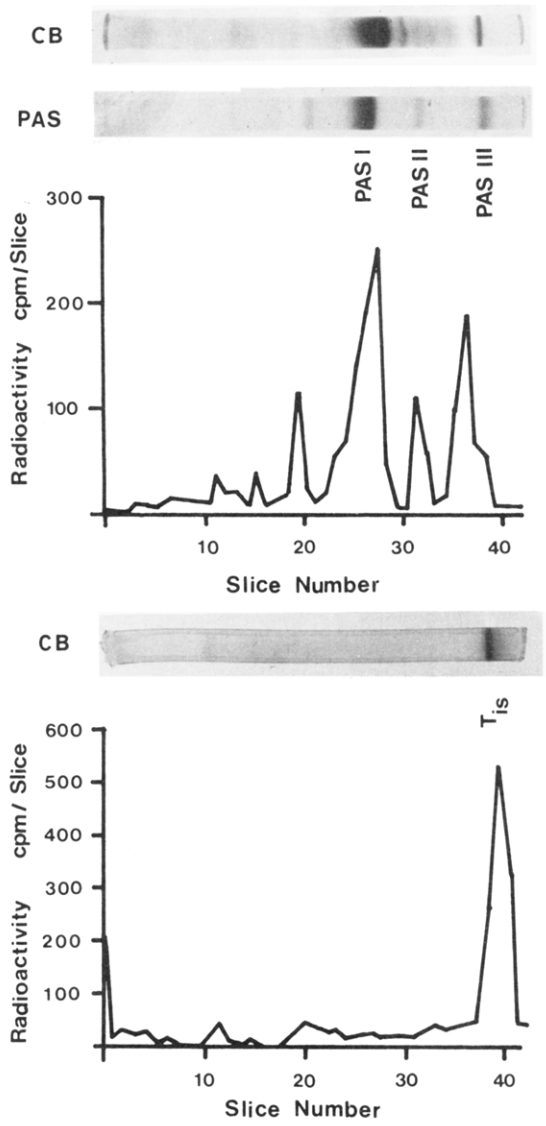

FIGURE 6: Electrophoretic profiles of glycophorin (upper half) isolated from ghosts labeled with [^{125}I]TID and purified as described in the text. Gels were stained with Coomassie Brilliant Blue R-250 (CB) or periodic acid–Schiff's reagent (PAS) (Fairbanks et al., 1971). Glycophorin was digested with trypsin (Furthmayr et al., 1978), and the insoluble Peptide T_{is} was electrophoresed on a discontinuous Tris–glycine system $(11.5 \times 2.7)^2$ as described previously (Brunner et al., 1979) (Lower half). For measurements of radioactivity, stained gels were cut into 1-mm slices.

subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (11.5×2.7).[2] The gel stained with Coomassie Blue showed one major band only migrating closely behind the tracking dye. This region contained 80% of the radioactivity while approximately 10–20% did not enter the separation gel. There was only background radioactivity along the remaining part of the gel.

Discussion

Upon photoactivation of [^{125}I]TID equilibrated with human erythrocyte membranes, lipids and intrinsic proteins became labeled while the amount of radioactivity incorporated by the peripheral proteins is exceedingly small. The distribution of radiolabel among the components of labeled ghosts is much different from the labeling patterns obtained with other hydrophobic, photoactivatable reagents (Bercovici & Gitler, 1978; Wells & Findlay, 1979a; Baylay & Knowles, 1980). At this stage of development, the present result indicates that radioiodinated TID is a useful reagent for labeling the hydrophobic core of membranes.

A principal goal of hydrophobic labeling of membranes is the identification of those domains of intrinsic proteins that are in contact with the lipid bilayer (Brunner, 1981). Several reagents have been described to selectively label the hydrophobic core. Although highly suggestive evidence has been presented for such claims, rigorous proof cannot be given until a more detailed knowledge of the structure of membrane proteins becomes available and analyses of labeled polypeptide segments can be performed to a resolution revealing the position of individual amino acids labeled in the polypeptide chain.

A strong argument for having achieved labeling selectivity is the absence of label in peripheral membrane proteins (and also in peripheral domains of integral proteins). As demonstrated in Figure 4 and Table II, none of the peripheral proteins of the red cell membrane (bands 1, 2, 4.1, 4.2, 5, and 6) incorporated significant amounts of radioactivity. Spectrin (bands 1 and 2), the major proteins of the membrane (~30%), contained as little as 0.2% of the radioactivity which was associated with proteins or approximately 0.01% of the total radiolabel originally present in the incubation medium. In contrast, in ghosts labeled with another carbene generator, [^{3}H]adamantanyldiazirine, ~5% of the radiolabel associated with proteins was found in spectrin (Bayley & Knowles, 1980). This labeling selectivity of TID must be related with the hydrophobicity of the reagent. Indeed, as shown in Table I, the partition coefficient (defined as the ratio of the concentration of reagent in the lipid phase of membranes to that in the aqueous buffer) of TID is larger than that of adamantanyldiazirine by 1 order of magnitude. The extent of labeling of peripheral proteins by a lipid-soluble reagent may, in addition, depend on specific or unspecific binding of reagent to certain hydrophobic areas on the protein surface. Furthermore, lifetime and chemical selectivity of the reactive species may play an important role. It is now recognized (Staros, 1980) that aryl nitrenes undergo intramolecular rearrangements which result in long-lived, electrophilic species. Even though these may be generated within the lipid core of a membrane, they may in turn diffuse out of the bilayer and preferentially react with nucleophiles believed to be located mainly at the water-exposed surface of proteins. A similar problem exists for adamantanyldiazirine which on photolysis yields the diazo isomer as the main product (Bayley & Knowles, 1978b). Diazoadamantane is a powerful electrophile and may also give rise to group-specific modification of externally located amino acid residues. This view is supported by the recent finding that labeling of red cell membranes with [^{3}H]adamantanyldiazirine resulted in a significant reduction of glycophorin labeling when in the aqueous buffer glutathione was present (Bayley & Knowles, 1980). Previous studies on the TID analogue, 3-(trifluoromethyl)-3-phenyldiazirine, showed that the corresponding diazo isomer is unreactive under the usual conditions of photolabeling procedures (Brunner et al., 1980). The unusual acid stability of diazo compounds containing an adjacent CF_3 group has also been utilized in another photolabeling reagent (Chowdhry et al., 1976). Thus, although TID is likely to photoisomerize to the diazo compound, the latter is predicted to be chemically unreactive under the pH conditions of the labeling process. Therefore, the likely absence of a long-lived, reactive species in the course of the photolysis of TID would eliminate a labeling pathway of peripheral proteins by similar mechanisms, as considered to be the case for aryl azides and adamantanyldiazirine, and this must

consequently result in an increased labeling selectivity.

A second observation which supports the view that TID reacts exclusively from within the lipid core is the lack of any detectable effect of reduced glutathione in the aqueous buffer (Table II). However, as the solvent water itself may be scavenging a carbene at diffusion-controlled rates, it seems unlikely that a reduction of the labeling extent would be detectable. Nevertheless, reduced glutathione would be predicted to compete with proteins for any hypothetical long-lived intermediate presumed to be reactive toward functional groups of the proteins.

As shown in Figure 3, by far most of the reagent reacted with the main components of the hydrophobic core, phospholipids and cholesterol. From this, we infer that proteins are labeled at sites which are in contact with the lipid bilayer. The most striking difference between these results and data derived from labeling experiments with adamantanyldiazirine and aryl azides is a far higher lipid to protein labeling ratio obtained with TID. In fact, the estimated values are approximately 16 for TID while those found for adamantanyldiazirine, and e.g., iodonaphthyl azide are 4 and 2, respectively. Baylay & Knowles (1980) have interpreted a high lipid to protein labeling ratio as indicative of a high reactivity of the photogenerated intermediate. However, this interpretation alone would not explain the obvious differences in the labeling patterns found with TID and adamantanyldiazirine, both assumed to give rise to high reactive carbenes.

It is quite possible that diazoadamantane-linked pathways significantly contribute to or even dominate the overall labeling process of proteins by adamantanyldiazirine. Indeed, diazo compounds react in a much more selective way than carbenes do, and some proteins having reactive residues at their surface may be efficiently derivatized by the former intermediate. Such group-specific pathways may well be responsible for the strong labeling of certain proteins (e.g., glycophorin). Similar ideas have been put forward by Hu & Wisnieski (1979) to rationalize the high efficiency of aryl nitrenes in labeling of membrane proteins. Again, glycophorin may be taken as an example, as approximately 10% of the radiolabel introduced as [^{125}I]iodonaphthyl azide (Kahane & Gitler, 1978) remained associated with this integral protein. This figure should be compared with the value of 0.2–0.3% found in this study. Since there is no evidence or reason to assume that in addition to the carbene other reactive species are formed, efficient site-specific modification presumably need not be considered. Indeed, this is compatible with the finding that protein labeling with TID was generally low.

In an attempt to further characterize labeled proteins, glycophorin was isolated from labeled red cell membranes and subjected to trypsin digestion. The "trypsin-insoluble" peptide T_{is} contained 95% of the radiolabel which was present in glycophorin, which demonstrates that the major sites of labeling lie within a peptide known from other evidence to span the bilayer (Jackson et al., 1973). Although useful information can be derived from such experiments, no clear-cut conclusions can be drawn concerning the disposition of the labeled amino acid residues until more detailed studies—including sequential degradation—have been completed. Recent labeling studies of brush-border membranes with [^{125}I]TID have given further evidence that labeling occurred within the lipid core (Spiess et al., 1982). Only that subunit of sucrase–isomaltase complex which interacts with the membrane bilayer was found to be labeled. Although very little radioactivity was incorporated into sucrase–isomaltase (<0.001% into sucrase; 0.011% into isomaltase), more than 90% of the radiolabel of the isomaltase was located in a hydrophobic polypeptide fragment which amounts to less than 3% of the protein mass.

[^{125}I]TID can be synthesized quite easily, and in particular, iodine can be introduced into the aromatic ring without difficulties and in a high yield. Thus, it should be possible to obtain TID with the same specific radioactivity as that of Na^{125}I (carrier-free commercial preparations have specific radioactivities as high as 2000 Ci mmol^{-1}) simply by reacting a mixture of the diazonium salt [3-(trifluoromethyl)-3-(*m*-diazophenyl)diazirine; see Experimental Procedures] with iodide. With little experience, the conversion of the stable intermediate X of Figure 1 to TID can be performed within 3 h. One problem is that for the purposes of autoradiography ^{125}I yields poor resolution because of the high γ radiation energy. If this would limit the usefulness of [^{125}I]TID as a labeling reagent, ^{131}I could be used instead (Teng & Chen, 1976). Since the radioactive reagent is available within hours, the inconvenience of a short half-life of ^{131}I (6 days) should not cause severe limitations.

At this stage of development, it appears that radiolabeled TID combines favorable properties which have not been met before in other hydrophobic photolabeling reagents. Work is now in progress to determine certain points such as specific or unspecific binding to protein or steric exclusion. Furthermore, since even carbenes do not react in a truly nonselective manner, it is possible that the distribution of label among the integral proteins does not in an absolute way reflect the ratio of the lipid-exposed surface areas which are accessible to the reagent. Proteins containing chemically inert residues only are likely to incorporate less radioactivity than those proteins which contain reactive groups buried in the bilayer. Finally, at the present time, no general methods are available to locate individual labeled amino acids in a polypeptide chain. When such methods become available, hydrophobic photolabeling will present a powerful tool to determine the folding patterns of integral membrane proteins.

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Electrostatic Control of Enzyme Reactions: Effect of Ionic Strength on the pK_a of an Essential Acidic Group on Glucose Oxidase†

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ABSTRACT: The dissociation constant of an essential acidic group on the reduced form of glucose oxidase from *Aspergillus niger* (K_4) has been found to be extremely sensitive to ionic strength. Increasing the ionic strength from 0.025 to 0.225 causes a decrease in p$K_{4,obsd}$ of 0.9 pH unit, from 8.2 to 7.3. Analysis of the ionic strength dependence of p$K_{4,obsd}$, making the assumption that the enzyme is a homogeneously charged impenetrable sphere [Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, Vol. 1, pp 282–289, 512–514, Academic Press, New York], predicts that the intrinsic pK_a of the acidic group is 6.7 and that the charge on the protein is −78. The enzyme was titrated from its isoelectric point (pH 4.05) to pH 7.7, the pH at which the ionic strength dependence was determined. It was found to have an actual charge at that pH of −77, in remarkable agreement with the theoretical prediction. Thus, glucose oxidase exerts electrostatic control on p$K_{4,obsd}$ as though it were a uniformly charged sphere. The group responsible for p$K_{4,obsd}$ has not been identified. However, its measured $\Delta H°_{obsd}$ of 8.0 kcal mol^{-1} and $\Delta S°_{obsd}$ of −6.1 cal mol^{-1} K^{-1}, together with its pK_a of 6.7, are consistent with the group being a histidine residue.

Electrostatic potential is an important contributor to the control of the activity of many sorts of enzymes: membrane-bound enzymes such as cytochrome oxidase (Maurel et al., 1978), enzymes such as ribonuclease and lysozyme which have polyelectrolytes as substrates (Irie, 1965; Maurel & Douzou, 1976), and acidic or basic enzymes which as a result of high surface charge create their own electrostatic potential (Valenzuela & Bender, 1971). Douzou & Maurel (1977) have reviewed the general mechanisms by which ionic control may be exerted on enzymatic reactions.

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is an acidic enzyme. Its reaction involves the apparent participation of a group whose dissociation occurs in a pH range in which the enzyme is polyanionic. Since fluctuations in ionic strength change the electrostatic potential exerted by the negative charge on the enzyme, they should alter the dissociation constant of this group. These fluctuations thus provide a mechanism for controlling the enzyme's activity.

The kinetics of the glucose oxidase reaction are easily analyzed in terms of individual rate constants according to pH-

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