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Synthesis of a photoactivable phospholipid containing an aromatic sulfonylazide and its interaction with proteins

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The photoactivable compound, fluorobenzenesulfonylazide (FBSA) was prepared and the structure and stability verified by gas chromatographic-mass spectral analysis, infrared spectroscopy and elemental analysis. The FBSA was reacted at the amino headgroup of ^{3}H-labeled phosphatidylethanolamine (PE) to produce the photoactivable phospholipid, ^{3}H-labelled 1,2-diacylglycero-3-(*N*-4-sulfonylazidophenylaminoethyl)phosphate at a yield of approx. 30%. Activation of the photoactivable phospholipid was accomplished with shortwave ultraviolet light as opposed to longwave ultraviolet light in which the sulfonylazide was unreactive. The [^{3}H]PE-benzenesulfonylazide was partitioned at nmol levels into low-density lipoprotein, high-density lipoprotein and retinal rod disc membranes. Photolysis experiments coupled with SDS-polyacrylamide gel electrophoresis fractionation showed the [^{3}H]PE-benzenesulfonylazide to crosslink to apolipoprotein B of low-density lipoprotein, apolipoprotein AI of high-density lipoprotein and rhodopsin of the disc membranes.

Introduction

For studies concerned with lipid-protein interactions, photoactivable phospholipids have been introduced as tools for probing specific domains of membrane proteins associated with phospholipid bilayers [1–4]. The commonly used photoactivable groups include aromatic azides, precursors for nitrenes and diazo or diazirines, precursors for carbenes. In a lipid-protein environment, both low and high efficiencies of crosslinking are known to occur [5,6]. In addition, a distinct preference for crosslinking to unsaturated or saturated hydrocarbons and specific amino acids has been reported [2]. Thus, it would seem appropriate to investigate a variety of different photoactivable lipids in order to limit nonuseful reactions and successfully probe protein domains in the hydrocarbon region as well as the lipid-aqueous interface.

In the present studies, we have chosen to use an aromatic sulfonylazide as the basic nitrene-generating probe. Sulfonylazides have been shown to exhibit high reactivity toward aromatic rings and to insert into primary, secondary and tertiary carbon-hydrogen bonds of alkanes [7–9]. Both thermolysis and photolysis have been utilized for initiating nitrogen release with subsequent nitrene

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Abbreviations: FBSA, fluorobenzenesulfonylazide; PE, phosphatidylethanolamine.

formation. In some photolysis studies, rearrangement has been shown to occur with aromatic sulfonylazides [10] and sulfonamide formation has been implicated as occurring from either hydrogen abstraction [11] or by cleavage of an initial insertion product [8].

In membrane-related studies, Sator et al. [12] have successfully used pyrenesulfonylazide in photolysis studies to label the acetylcholine receptor of electroplax membranes. In addition, pyrenesulfonylazide has been found to be a useful fluorescent marker for crosslinking to apoprotein B of low-density lipoproteins (Iwanik, M.J. and Shaw, J.M., unpublished data). In the present work, we have modified the headgroup of [^{3}H]PE by covalently attaching a phenylsulfonylazide. The photoactivable phospholipid has been partitioned into purified lipoproteins and retinal rod disc membranes. Photolysis studies coupled with SDS-polyacrylamide gel electrophoresis revealed crosslinking to the major apoproteins of the lipoproteins and rhodopsin of the disc membranes.

Materials and Methods

Synthesis of FBSA. The method of preparation of FBSA represents a slight modification of the procedure described by Dermer and Edmison [13] and Leffler and Tsuno [14]. Fluorobenzenesulfonylchloride, 1 g, was dissolved in 12 ml acetone, cooled on ice then 10 ml cold acetone containing 1.68 g sodium azide was added. An additional 20 ml deionized water at 4°C was added and the reaction was stirred overnight on ice. Following the addition of cold water to the reaction mixture, FBSA separated out as an oil. Hexane at 4°C was utilized to increase the volume of the oil phase and the two phases divided in a separatory funnel. The aqueous-acetone phase was washed with cold hexane and the pooled hexane-oil fractions dried under vacuum to remove solvent. Under the reaction conditions, fluorobenzenesulfonylchloride is completely converted to FBSA. The mol% recovery of FBSA from the original fluorobenzenesulfonylchloride was approx. 79%, as determined by recording the weights of starting material and product. The FBSA was stored at 4°C as an oil in the dark. Elemental analysis of the synthesized FBSA was performed by Galbraith laboratories, Knoxville, TN.

Gas chromatographic-mass spectral analysis and infrared studies. FBSA in hexane (1–2 mg/ml) was examined on a Finnigan 4000 gas chromatograph-mass spectrometer 6110 data system. The gas chromatographic separation was carried out on a 91 cm × 2 mm glass column containing 3% OV-17 at a column temperature of 120°C isothermal, with a helium flow of 20 ml/min. The mass spectrometer conditions were electron ionization with a separation temperature of 220°C, a source temperature of 260°C, and ionization potential of 70 eV.

FBSA was examined by infrared analysis as a film made by mixing the oil with KBr. Fluorobenzenesulfonylchloride was dissolved in chloroform and then added to KBr with a film formed after evaporation of the chloroform. Spectra were recorded on a Perkin-Elmer model 700 between frequencies of 650 and 4000 cm^{-1}.

Preparation of [^{3}H]PE-benzenesulfonylazide. Phosphatidylethanolamine (500 mg, Avanti) was tritiated by Amersham using tritium gas (10 Ci) and platinum oxide (prereduced) at 25°C under argon in ethanol/hexane. The [^{3}H]PE with approx. 50% of all double bonds reduced was shipped on dry ice to our laboratory after preparation. The ethanol/hexane was removed by vacuum rotoevaporation and the [^{3}H]PE was dissolved in spectral-grade chloroform containing 0.75% ethanol. The [^{3}H]PE (500 mg at 800–1300 mCi/mmol) in chloroform was fractionated on a 2.5 × 15 cm silicic acid column (Silica gel 60, E. Merck) with increasing percentages of methanol in chloroform. The [^{3}H]PE fractions after column chromatography were further purified on silica-gel thin layers (Silica gel 60, 0.25 mm, E. Merck) using the solvent system, chloroform/methanol/water (65 : 25 : 4 v/v), then stored at −70°C in spectral-grade chloroform containing 0.75% ethanol.

The [^{3}H]PE (0.65 μmol) in chloroform was placed in a 2-ml Pierce reactivial and the solvent was evaporated under nitrogen or argon. FBSA (60 μmol) was added to the vial followed by 300 μl spectral chloroform (maintained over 4-Å molecular seives), then 2.5 μl triethylamine (18 μmol). The reaction mixture was stirred for 3 days at 37°C under argon, then fractionated on a silicic acid column (Silica gel 60, 230–460 mesh, E. Merck) with increasing concentrations of methanol

in chloroform. Aliquots from column fractions were counted for tritium and peak areas pooled and measured for phosphorus using the Bartlett procedure [15]. The new peak corresponding to [^{3}H]PE-benezenesulfonylazide was further fractionated on silicic acid thin layers using the solvent system chloroform/methanol/water (65 : 25 : 4, v/v). The [^{3}H]PE-benzenesulfonylazide in chloroform was passed through 0.2-μm polytetrafluoroethylene filters (Schleicher and Schuell, TE-35) and stored in the dark at -20°C. The radiospecific activity of the [^{3}H]PE-benzenesulfonylazide was on the order of 0.4 μCi/nmol phosphorus.

Isolation of lipoproteins and retinal rod discs. Low-density lipoprotein (density 1.006–1.063 g/ml) and high-density lipoprotein (density 1.063–1.215 g/ml) were isolated by density flotation using KBr, as originally described by Havel et al. [16]. Details of the fractionation using argon-purged buffers and additional flotation wash steps have been described [17]. The lipoproteins were fractionated on a SDS-polyacrylamide slab gel (5–15% exponential) with 4.5% stacking gel using the buffers described by Laemmli [18].

The retinal rod disc membranes were a gift of Dr. Burton Litman, University of Virginia. The disc membranes were prepared as described by Smith et al. [19] and maintained in the dark at -20°C in 50 mM Tris-acetate buffer (pH 7.0). SDS-polyacrylamide gel electrophoresis of the retinal rod discs was performed using the same gel and buffer system described for the lipoproteins. The retinal rod discs were solubilized in SDS but no heat or mercaptoethanol was utilized in preparing the samples.

Partition of [^{3}H]PE-benzenesulfonylazide into lipoproteins and retinal rod discs. The [^{3}H]PE-benzenesulfonylazide (10 nmol, 4 μCi) was vigorously mixed in a narrow Pierce reactivial with 20 μl 100% ethanol. 2 μl [^{3}]PE-benzenesulfonylazide (1 nmol) was injected with a Hamilton syringe into a solution of low-density lipoprotein, high-density lipoprotein (100 μg protein) or retinal rod discs (100 μg rhodopsin) in a volume of 100 μl. The mixture was incubated for 30 min to 1 h with stirring by a magnetic flea. The resulting complexes were irradiated or maintained in the dark and immediately analyzed by SDS-polyacrylamide gel electrophoresis. Partition experiments with high-density lipoprotein were performed with up to 15 nmol [^{3}H]PE-benzenesulfonylazide while maintaining the percentage of ethanol (approx. 2%) and protein (100 μg) constant.

Irradiation of FBSA and [^{3}H]PE-benzenesulfonylazide samples. The FBSA and [^{3}H]PE-benzenesulfonylazide was resuspended in cyclohexane in a 1 cm $\times$ 1 cm quartz cuvette and exposed to intensity-filtered longwave (approx. 366 nm) or shortwave (approx. 254 nm) light from a Model UVSL-58 lamp (Ultra-Violet Products). With filter in place, the intensity of shortwave light was 1600 μW/cm^2 and that of the longwave light 2000 μW/cm^2 at a distance of 3 cm. The model UVSL-58 lamp filter removed approx. 33% of longwave and approx. 43% of shortwave light intensity. The samples were exposed to irradiation for 5–30-min periods at a distance of 2–3 cm, then examined immediately for spectral absorption between 200 and 400 nm in cyclohexane using a Cary 210 double-beam spectrophotometer. Samples were examined by thin-layer chromatography after irradiation using the solvent system, chloroform/methanol/water (65 : 25 : 4, v/v). The [^{3}H]PE-benzenesulfonylazide-lipoprotein or retinal rod disc complexes were irradiated on ice in quartz tubes for 10-min time periods with either filtered, shortwave or longwave ultraviolet light.

Results and Discussion

Synthesis of FBSA

Fluorobenzenesulfonylazide has been prepared by established procedures as a photoactivable compound for covalent attachment to the amino headgroup of PE, radiolabeled in the fatty acyl chains. FBSA was synthesized from fluorobenzenesulfonylchloride and sodium azide in acetone/water and collected as an oil. The utilization of acetone/water was preferred over methanol or ethanol since gas chromatograph peaks accounting for as much as 14% of the sample contained molecular ion fragments corresponding to the methyl or ethylsulfonyl ester after mass spectral analysis. This observation was first reported by Zalkow and Oehlschlager [20] who found as much as 25% ethylbenzenesulfonate if benzenesul-

fonylchloride was first dissolved in ethanol. Both gas chromatographic and mass spectral analyses were performed for FBSA synthesized in acetone/water. Gas chromatography revealed in addition to the solvent peak, hexane, only one peak with a retention time of 1.30 min. The mass spectrum of the peak at 1.30 min shows three readily identifiable molecular ions, FBSA (*m/z* 201), a fluorobenzenesulfonyl ion (*m/z* 159) and a fluorophenyl ion (*m/z* 95) (Fig. 1). The ion (*m/z* 75) likely represents a C_6H_3 ion resulting from the loss of HF from the fluorophenyl ion (C_6H_4F, *m/z* 95). No molecular ion for fluorobenzenesulfonylchloride (*m/z* 194) is present. Fluorobenzenesulfonylchloride was observed to elute from the gas chromatograph with a retention time of 0.97 min.

The synthesized FBSA showed the characteristic sulfonylazide signal at 2130 cm^{-1} when examined by infrared analysis. A quantitative microanalysis of the synthesized compound showed 35.64% carbon, 2.11% hydrogen, 20.95% nitrogen, 9.29% fluorine, 15.84% sulfur, 15.95% oxygen and 0.04% chlorine. A theoretical elemental analysis of FBSA is 35.82% carbon, 2.00% hydrogen, 20.89% nitrogen, 9.44% fluorine, 15.94% sulfur, 15.91% oxygen and 0.00% chlorine. The results of elemental analysis corresponded to the compound FBSA with negligible contamination by fluorobenzenesulfonylchloride.

Photolysis of FBSA

Photolysis of FBSA in cyclohexane was carried out using either longwave or shortwave ultraviolet light, then the absorption spectra of the compound was recorded (Fig. 2). FBSA showed no sensitivity to longwave ultraviolet light for as many as six, 5-min exposures (Fig. 2A). The absorption at 220 nm for FBSA rapidly decreased after sequential 5-min exposures to shortwave ultraviolet light (Fig. 2B). At 10-min exposures, the majority of the sulfonylazide signal was reduced, therefore this time period was chosen as an optimal photolysis time. The photolysis of FBSA in cyclohexane using shortwave ultraviolet light followed by thin-layer chromatography using hexane/ether/acetic acid/water (60 : 40 : 1 : 1, v/v) resulted in multiple compounds with R_f values of 0.83, 0.69, 0.20, 0.14 and 0.00, whereas nonirradiated FBSA showed a R_f value of 0.70 and fluorobenzenesulfonylchloride, 0.78. The FBSA sensitivity to shortwave ultraviolet light with no activation of the sulfonylazide by longwave regions may prove useful in combination experiments with photolytic compounds which are activated at longer wavelengths. Consequently, crosslinking could be performed by first activation with longwave ultraviolet light followed by photolysis of the benzenesulfonylazide using shortwave ultraviolet light.

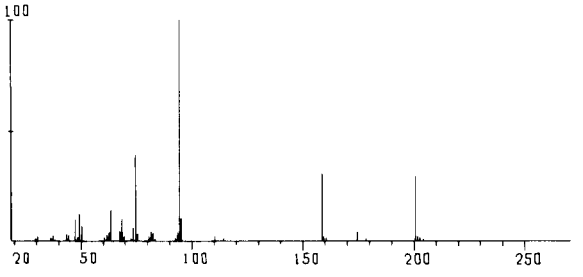

Fig. 1. Mass spectral analysis of FBSA. FBSA (1–2 mg/ml) in hexane eluted from the gas chromatograph as one peak at 1.30 min. The mass spectrum of the FBSA peak is shown with the *x*-axis representing the mass/charge ratio (*m/z*) and the *y*-axis the relative abundance of the molecular ion or fragment.

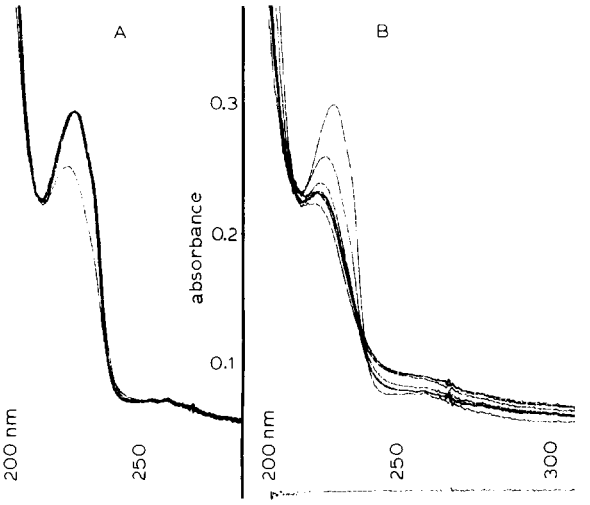

Fig. 2. FBSA (22 μg) in 2 ml cyclohexane was initially scanned in a double-beam spectrophotometer (upper peaks A and B). FBSA was irradiated with filtered longwave ultraviolet light (A) or filtered shortwave ultraviolet light (B) at 5-min intervals from a distance of 2 cm. The samples were rescanned after each irradiation period. The lower peak in (A) represents irradiation with shortwave ultraviolet light after completion of irradiation periods with longwave ultraviolet light.

Coupling FBSA to the amino group of [^{3}H]PE

The reaction of FBSA with [^{3}H]PE was carried out at 37°C in dry chloroform in the presence of triethylamine in analogous fashion for coupling fluoronitrobenzeneazide to the amino group of phosphatidylethanolamine [21]. The reaction mixture was fractionated on a silicic acid column and the radiolabeled fractions were collected and counted (Fig. 3). The appearance of a new peak representing 30% of total cpm eluted in approx. 15% methanol in chloroform and was further fractionated on silicic acid thin layers. The compound migrated on silica-gel thin layers as one spot with a higher R_f (0.59) than [^{3}H]PE (0.50) in the solvent system, chloroform/methanol/water (65 : 25 : 4) and the compound stained for phosphorus. Photolysis of the [^{3}H]phospholipid in cyclohexane was examined spectrophotometrically after subtracting out the absorption due to [^{3}H]PE alone in the 210–300 nm region. A peak at 220 nm (sulfonylazide) showed light sensitivity analogous to FBSA.

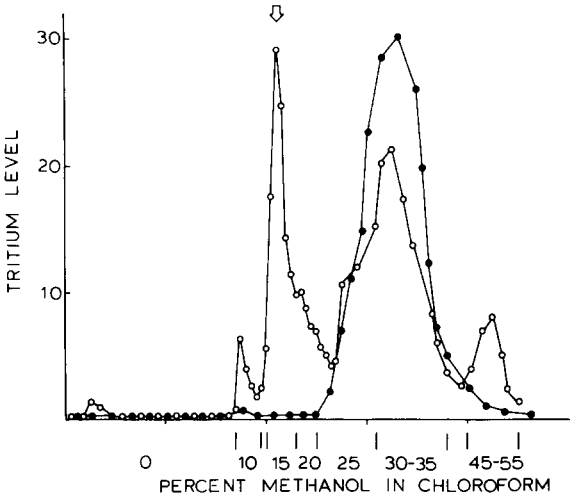

Fig. 3. Silicic acid column chromatography of [^{3}H]PE-benzenesulfonylazide. The [^{3}H]PE/FBSA reaction mix was applied to a 2.5 × 15 cm silicic acid column (○). The column was washed extensively with chloroform followed by increasing percentages of methanol in chloroform. Aliquots of column fractions were counted for tritium (relative scale, *y*-axis). The peak-area corresponding to the arrow represents [^{3}H]PE-benzenesulfonylazide (○). The peaks at approx. 10% and approx. 50% methanol in chloroform are produced during the 37°C incubation and represent ^{3}H-free fatty acid and lyso[^{3}H]PE, respectively (○). Purified [^{3}H]PE not incubated at 37°C was fractionated and is illustrated in the figure (●).

Fractionation of the photolyzed lipid on silicic acid thin layers reduced and streaked the radiolabeled peak over a broader area relative to control photoactivable [^{3}H]phospholipid. The benzenesulfonylazide containing [^{3}H]phospholipid was shown to possess the same ^{3}H-labeled acyl chain/phosphorus radiospecific activity as the starting [^{3}H]PE. The only expected product from the coupling reaction which contains 2 acyl chains/1 phosphorus and shows the presence of the light-sensitive sulfonylazide group is [^{3}H]1,2-diacylglycero-3-(*N*-4-sulfonylazidophenylaminoethyl)-phosphate. Other photoactivable derivatives coupled to the amino headgroup of [^{3}H]PE can be readily prepared [21,22] although most attention has been focused on phospholipids labeled in the acyl chain with photoactivable groups [23]. The work of Ross et al. [3] with glycophorin A in reconstituted bilayers, however, suggests that headgroup-labeled phospholipids may be preferable for labeling peptide regions close to the bilayer/aqueous interface.

Photolysis studies of [^{3}H]PE-benzenesulfonylazide partitioned into retinal rod disc membranes and human lipoproteins

To test the effectiveness of [^{3}H]PE-benzenesulfonylazide as a crosslinking agent, three different biological lipid-protein systems have been examined which contain proteins known to interact strongly with lipids. These include the purified lipoproteins, high-density lipoprotein and low-density lipoprotein and retinal rod disc membranes. Rhodopsin, the major protein in retinal rod discs, is thought to form α-helices perpendicular to the plane of the membrane much like bacteriorhodopsin [24] and interact mainly with phospholipid since the disc membrane contains low levels of cholesterol. Apoprotein AI of high-density lipoprotein contains regions of amphipathic helices which apparently interact with phospholipid-cholesterol at surface regions of the high-density lipoprotein particle [25,26]. Apoprotein B of low-density lipoprotein interacts strongly with phospholipid-cholesterol and/or cholesterol ester-triacylglycerol domains.

Probe levels of the [^{3}H]PE-benzenesulfonylazide, 1 nmol total phosphorus, were partitioned into each of the experimental systems representing

100 μg protein. At these levels of [^{3}H]PE-benzenesulfonylazide, 1 nmol photoactivable phospholipid is present for every 70–400 nmol native lipid in the lipoprotein or disc membrane. Photolysis of each sample was accomplished with a 10-min exposure to shortwave ultraviolet light. The lipoproteins or retinal rod discs were solubilized using SDS and 25 μg protein fractionated on an exponential polyacrylamide slab gel (Fig. 4). Less than 2% of the total [^{3}H]PE-benezenesulfonylazide crosslinked to rhodopsin of the native retinal rod disc membrane (Fig. 4A). By knowing the molecular weight of rhodopsin (approx. 37000), we calculate that 1 nmol [^{3}H]PE-benzenesulfonylazide is crosslinked for every 135 nmol rhodopsin. For 100 μg high-density lipoprotein, approx. 2% of the total ^{3}H-labeled lipid utilized in the experiment is crosslinked to apoprotein AI (Fig. 4B). Apoprotein AI is a M_r 28000 protein and the photolytic crosslinking occurs at a ratio of 120 nmol apoprotein AI for every 1 nmol [^{3}H]PE-benzenesulfonylazide. For low-density lipoprotein, approx. 6% of the total cpm crosslink to apoprotein B (Fig. 4C). Assuming a molecular weight of 250000 for apoprotein B [27], a ratio of 1 nmol [^{3}H]PE-benzenesulfonylazide to 8 nmol apoprotein B is crosslinked. In general, the 2–6% crosslinking of photoactivable lipid to protein probably reflects the low efficiency of the covalent insertion reactions. In none of the photolysis studies could apoproteins or rhodopsin be crosslinked when using longwave ultraviolet light. In addition, the SDS-polyacrylamide gel patterns of the apolipoproteins or rhodopsin suggest no unusual alteration of the protein by the 10-min exposures to low-intensity shortwave ultraviolet light. 1-h exposures, however, can lead to mild fragmentation of apoprotein B and some multiplicity of protein bands in fractionated fibroblast plasma membranes (unpublished data). Consequently, long-term exposures of any system to low-intensity shortwave ultraviolet light should be avoided.

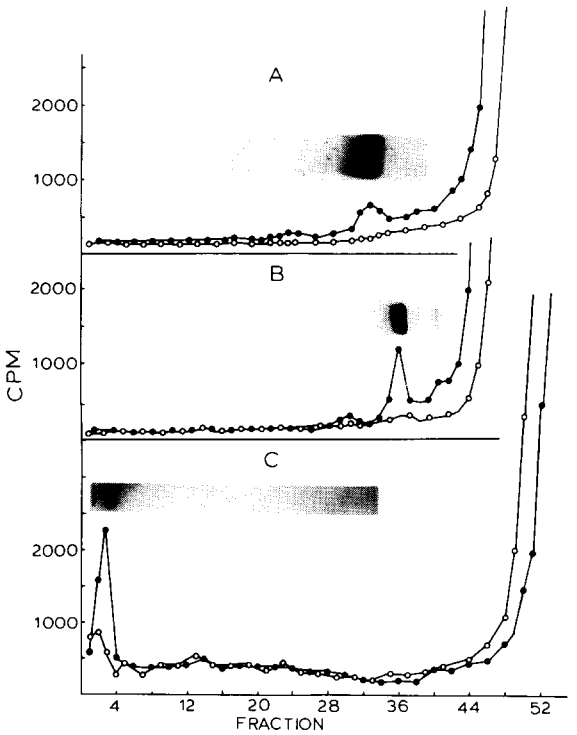

Fig. 4. SDS-polyacrylamide gel electrophoresis of irradiated [^{3}H]PE-benzenesulfonylazide-lipoprotein and retinal rod disc complexes. [^{3}H]PE-benzenesulfonylazide (0.25 nmol, 65000 cpm) complexed to retinal rod discs (A, 25 μg rhodopsin), high-density lipoprotein (B, 25 μg protein) or low-density lipoprotein (C, 25 μg protein) were solubilized using SDS and fractionated by SDS-polyacrylamide gel electrophoresis. Gel slices were counted for radioactivity after standing 24 h in 2% SDS. (●, Samples irradiated for 10 min; ○, nonirradiated samples. An irradiated sample after gel fractionation was stained using Coomassie blue and is shown as an inset. The insets are not to the same scale as the plots.

By increasing the amounts of [^{3}H]PE-benzenesulfonylazide partitioned into protein-lipid system, higher levels of photoactivable lipid can be crosslinked to protein. This is illustrated for high-density lipoprotein in Fig. 5. Following photolysis and polyacrylamide gel fractionation, an almost linear increase in [^{3}H]PE-benzenesulfonylazide was observed to be crosslinked per μmol apoprotein AI. A 10-fold increase in the ratio of ^{3}H-labeled lipid crosslinked to apoprotein AI was observed at the upper level of ^{3}H-labeled lipid resulting in 1 nmol ^{3}H-labeled lipid crosslinked for every 20 nmol apoprotein AI. The relatively linear increase in crosslinking up to 15 nmol ^{3}H-labeled lipid suggests that lipid domains remain accessible to the partition of [^{3}H]PE-benzenesulfonylazide into high-density lipoprotein and that peptide sites for crosslinking have not been saturated. In addition, isolation of sufficient quantities of crosslinked products for analysis can be made simpler by selecting maximal levels of photoactivable phos-

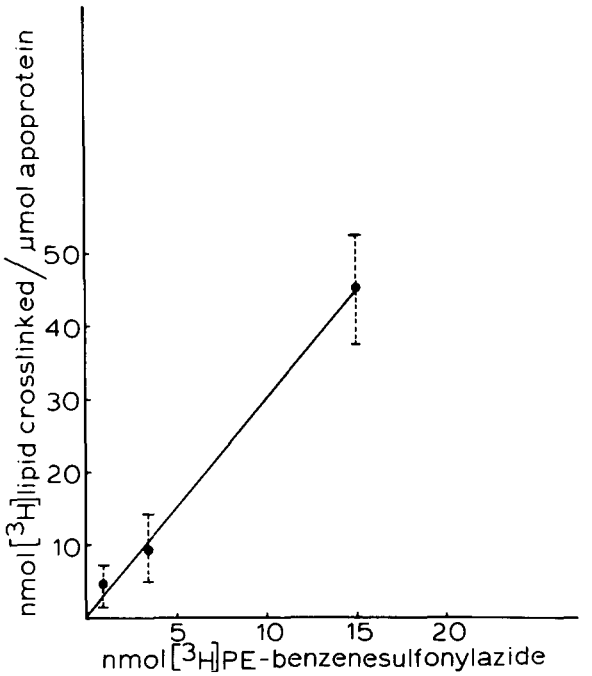

Fig. 5. Concentration dependence of [^{3}H]PE-benezenesulfonylazide crosslinking to apoprotein AI of high-density lipoprotein. Increasing concentrations of [^{3}H]PE-benzenesulfonylazide were partitioned into high-density lipoprotein (100 μg protein). Irradiated or nonirradiated samples were fractionated by SDS-polyacrylamide gel electrophoresis and the nmol [^{3}H]PE-benzenesulfonylazide crosslinked to apoprotein AI was determined.

pholipid for partition into lipoprotein. A determination of the peptide regions and specific amino acids crosslinked in apoprotein AI should enable us to establish the degree of crosslinking specificity of benzenesulfonylazide phospholipids.

Acknowledgements

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