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# Sugar-Mediated Crosslinking of α-Biotinylated-Lys to Cysteamine-Agarose Support

*A Method to Isolate Maillard Lys-Lys-Like Crosslinks*

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## Abstract

Advanced glycation end products (AGEs) and, specifically, protein-protein AGE crosslinks have long been studied for their potential role in aging, diabetic complications and Alzheimer disease. With few exceptions, the chemical nature of these structures remains unknown. We report here a simple approach that allows the preparation and isolation of milligram quantities of sugar-mediated AGE Lys-Lys-like crosslinks from glycation mixtures. The method is based on a sugar-dependent incorporation of $N^{\alpha}$-biotinyl-L-Lys into cysteaminyldisulfide Sepharose 6B (AE-S-S-Sepharose 6B). Glycation mixtures with six different sugars showed a time- and sugar-dependent decrease in the concentration of the support-bound primary amino groups and accounted for almost 90% loss of cysteaminyl amino groups at the end of the various incubation periods. 4-Hydroxyazobenzene-2-carboxylic acid–avidin assays indicated the incorporation of $N^{\alpha}$-biotinyl-L-Lys equal to 8% of the total support amino groups with methylglyoxal after 7 d and 1% with fructose and glucose after 1 mo of incubation. Treatment of the washed, sugar-modified supports with 2-mercaptoethanol released the bulk of the bound AGE modifications and the crosslinks. Subsequent fractionation of these preparations over a monomeric avidin column afforded a complete separation of sugar-mediated AGE modifications and the crosslinks. Depending on the sugar employed, micromolar amounts of biotinylated Lys-Lys-like crosslinks were generated by this two-step procedure from 8 mL of the original AE-S-S-Sepharose 6B.

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**Index Entries:** Crosslinks; Maillard reactions; advanced glycation end products; 2-aminoethyldisulfide sepharose 6B; $N^{\alpha}$-biotinyl lysine.

## Introduction

The Maillard reaction between reducing sugars and protein amino groups had long been suggested to be one of the key factors contributing to the development of age-related and diabetic changes associated with long-lived proteins such as lens crystallins and collagen *(1–3)*. The initial reaction involves the nonenzymatic condensation of carbonyl compounds with amino groups of proteins to form the initial ketoamines or Amadori products. These adducts undergo a series of reactions during relatively prolonged periods of time, which ultimately lead to the formation of advanced glycation end products (AGEs) *(3–5)*. AGEs are a relatively heterogeneous group of ill-characterized compounds that includes both lysine modifications (e.g., CML, CEL) and protein-protein crosslinks (e.g., pentosidine, MOLD, GOLD, vesperlysines), all of which are found in elevated quantities on posttranslationally modified proteins from human aged and pathologic tissues and body fluids *(6–12)*. The formation of fluorophores and yellow chromophores in aged and cataractous lens may represent such Maillard-derived AGEs *(6–8,13–17)*.

For the past decade researches have attempted to purify and characterize the AGE crosslinks, but only six minor adducts have been isolated: pentosidine *(6)*, vesperlysine A *(7,8,11)*, MOLD *(9)*, GOLD *(10)*, threosidine *(18)*, and furopyrrolopyridine *(19)*. With the exception of GOLD and MOLD crosslinks, the other four structures are fluorescent and are present in minute amounts in even advanced stages of cataract or uremia *(6–10,18,19)*. The isolation of sufficient amounts of intact purified material for performing structural work was shown to be a major challenge encountered by different groups in determining the structure of these crosslinks. In addition, since most of the crosslinks are acid labile *(20)*, it is quite possible that these crosslinks represent <1% of total AGE crosslinks to be isolated *(20)*. Therefore, acid hydrolysis of crosslinked proteins, often used to isolate the AGEs *(6–10,18,19)*, has rather limited use and may also lead to artifacts *(21)*. The synthesis of crosslinks from $N^{\alpha}$-blocked Lys or Arg and sugars generates a mixture of advanced glycation products and complex sugar condensation products from which crosslinks cannot be easily identified or separated *(6–10,18–20)*.

Keeping this in mind, we undertook the development of a simple and reliable method for the preparation of AGE crosslinks under physiologic conditions. Such a method could be used to generate Lys-Lys-like crosslinks with any sugar and to purify them under conditions that would not alter their structure. We explored the possibility of using cysteaminyldisulfide Sepharose (AE-S-S-Sepharose) for glycation-related applications, because this matrix contains in a relatively high abundance the amino groups uniformly attached to support through a disulfide bond. Because the resin is relatively stable in solutions for prolonged periods under physiologic con-

ditions, we attempted to crosslink the amino groups of this support to a Lys residue that contains a biotinyl group in the alpha-position. The sugar-mediated incorporation of the *N*α-biotinyl-Lys donor to amino groups of cysteaminyl disulfide Sepharose 6B permitted isolation of the crosslinked species by monomeric avidin chromatography. Because the conditions employed for the purification of these compounds are relatively mild, it allowed an isolation of unaltered Lys-Lys-like crosslinks in appreciable amounts for possible structural characterization in the future.

## Materials and Methods

### *Reagents and Equipment*

Thiopropyl Sepharose 6B was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). AF-Amino-650M Toyopearl was from TosoHaas (Montgomeryville, PA). UltraLink immobilized monomeric avidin on 3M Emphaze was purchased from Pierce (Rockford, IL). Dithiothreitol (DTT) was obtained from Boehringer Mannheim (Indianapolis, IN). Avidin was from Molecular Probes (Eugene, OR). 2-Aminoethanol, d-Biotin, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), guanidine hydrochloride, guanidine thiocyanate, 4-hydroxyazobenzene-2-carboxylic acid (HABA), a mixture of standard amino acids, 2-mercaptoethanol (2-MeSH), were obtained from Sigma (St. Louis, MO). Thioctic acid was from Aldrich (Milwaukee, WI). (2-Aminoethyl)methane thiosulfonate hydrobromide (2-AE-MTS) was purchased from Toronto Research Chemicals (Toronto, Canada). All the sugars used in this work were the highest quality (>99%) available from Fluka (Milwaukee, WI). All other reagents were the highest analytical quality available from Fisher (Pittsburgh, PA) or ACROS (Pittsburgh, PA). Syringe-less filter devices (GMF membrane, 0.45-µm pores) Autovial 5 were obtained from Whatman (Clifton, NJ). All the buffers were Chelex treated *(22)* and degassed by sonication for at least 15 min before use. Deionized water of Milli Q quality was used throughout all the experiments.

### *Synthesis of Nα-Biotinyl-L-Lysine*

The procedure for *N*α-biotinyl-L-lysine (isobiocytin) preparation was similar to a protocol for biocytin synthesis *(23)*. *N*ε-(*t*-butyloxycarbonyl)-L-lysine (0.80 g, 3.25 mmol) was dissolved in a mixture of 15 mL of pyridine, 6 mL of water, and 2 mL of 1 *N* NaOH. After solubilization, biotin *p*-nitrophenyl ester (1.0 g, 2.74 mmol) *(23)* and 2 mL of 1 *N* NaOH were added, the resulting mixture was stirred for 30 min, and 1 mL of 1 *N* NaOH was added with constant stirring. After 1.5 h, pyridine was distilled off in vacuo at 40°C, and the remaining solution was filtered, diluted by water to 20 mL, cooled to 0°C overnight, and crystalline sodium *p*-nitrophenolate filtered off. The filtrate was carefully acidified at 5°C with 0.1 *N* HCl until reaching pH 2.0 and allowed to stand at this temperature overnight for crystallization. The crystalline *N*α-biotinyl-*N*ε-(*t*-butyloxycarbonyl)-L-lysine

was separated, washed with cold water, and dried in vacuo over $CaCl_2$. Yield: 1.06 g (76%). Anal. Calcd. for $C_{21}H_{36}N_4O_6S \times 2H_2O$: C, 49.6; H, 7.9; N, 11.0; S, 6.3. Found: C, 50.2; H, 7.6; N, 11.0; S, 6.5.

$N^{\alpha}$-Biotinyl-$N^{\varepsilon}$-(*t*-butyloxycarbonyl)-L-lysine (1.9 g, 3.73 mmol) was deprotected in 15 mL of 97% formic acid for 1 h at room temperature, and the formic acid was removed in vacuum at 40°C with repeated additions of water. The crystalline residue was dissolved in 15 mL of water, 125 mL of acetone was added, and the solution was allowed to crystallize at 2°C for 10 h. The crystals were separated, washed with acetone, and dried in vacuo over $CaCl_2$. The yield of crystalline isobiocytin was 1.15 g, with an additional 0.21 g obtained from the mother liquors (93% overall); M.p. 237–239°C (dec.); $[\alpha]_D^{25} = +44.3°$ (c = 1.0, water). Major nonsolvent peak in FAB MS: 373. Exact mass of the $[M + H]^+$ ion: The mass calculated for $C_{16}H_{29}N_4O_4S$ was 373.1910; found: 373.1910. Anal. calcd. for $C_{16}H_{28}N_4O_4S \times H_2O$: C, 49.2; H, 7.7; N, 14.4; S, 8.2. Found: C, 48.4; H, 7.8; N, 14.1; S, 8.3.

Tentative assignments in the nuclear magnetic resonance (NMR) spectra were done using published data for biotin *(24)*. $^{13}C$ NMR: [ppm rel. TSP standard]: 25.03, 27.88, 29.18, 30.41, 30.65, 33.93 ($3C_{Lys}$,$3C_{Biot}$), 38.09 ($C\alpha_{Biot}$); 42.02 ($C\varepsilon_{Lys}$), 42.53 ($C5_{Biot}$); 57.56 ($C\alpha_{Lys}$), 58.22 ($C2_{Biot}$), 63.01 ($C4_{Biot}$); 64.83 ($C3_{Biot}$); 168.03 ($=[NH]_2CO_{Biot}$); 178.82 ($CONH_{Biot}$); 181.70 ($COOH_{Lys}$). $^1H$ NMR: [ppm rel. TSP standard; *J*, Hz] 4.616 (dd, $H4_{Biot}$, $J_{3,4} = 8.0$, $J_{4,5A} = 4.8$), 4.433 (dd, $H3_{Biot}$, $J_{2,3} = 4.5$), 4.177 (dd, $H\alpha_{Lys}$, $J_{\alpha,\beta A} = 8.3$, $J_{\alpha,\beta B} = 5.0$), 3.345 (m, $H2_{Biot}$), 3.006 (t, $2H\varepsilon_{Lys}$, $J_{\delta,\varepsilon} = 7.6$), 3.003 (dd, $H5A_{Biot}$, $J_{5A,5B} = -13.0$), 2.787 (d, $H5B_{Biot}$), 2.326 (dt, $H\alpha A_{Biot}$, $J_{\alpha A,\beta} = 7.5$, $J_{\alpha A,\alpha B} = -14.3$), 2.324 (dt, $H\alpha B_{Biot}$, $J_{\alpha B,\beta} = 7.1$), 1.82 (m, $H\delta A_{Biot}$), 1.75–1.57 (m, $H\delta B_{Biot}$, $2H\delta_{Lys}$, $2H\beta_{Biot}$, $2H\beta_{Lys}$), 1.42 (m, $2H\gamma_{Biot}$, $2H\gamma_{Lys}$).

### *Synthesis of Aminoethyl Disulfide Sepharose 6B*

The preparation of the free thiol form of Thiopropyl Sepharose 6B was carried out essentially as recommended by the manufacturer. Fifteen grams of the support was usually processed each time for the preparation of aminoethyl disulfide Sepharose 6B (AE-S-S-Sepharose 6B). Completeness of the support reduction was verified spectrophotometrically by monitoring the DTT-mediated release of 2-thiopyridone into the supernatant at $\lambda = 343$ nm ($\varepsilon_{343} = 8.08 \times 10^3\ M^{-1}/cm$). Usually, the complete reduction of the support was attained within 1 h of incubation at room temperature. At the end of the incubation period, the slurry was transferred onto a medium-frit sintered glass funnel and washed thoroughly with an aqueous 20% ethanolic solution followed by a wash with 0.2 *M* $NaHCO_3$; 1.0 m*M* EDTA, pH 8.3. The slurry was dried to a "wet cake" and transferred to a beaker. The volume of the slurry was adjusted to 400 mL with 0.2 *M* $NaHCO_3$, 1.0 m*M* EDTA (pH 8.3), and the pH of the solution was adjusted to 8.3 with diluted acetic acid.

AE-MTS (1.32 g, 5.6 mmol) was added in small portions to the slurry with gentle stirring. Since during the course of the reaction between SH-groups of the support and 2-AE-MTS methanesulfonic acid is released

*(25)*, the pH of the reaction mixture was kept close to 8.0–8.2 by titration of the solution with 10% $NH_4OH$ simultaneously with the addition of 2-AE-MTS. The slurry was incubated for another hour with gentle stirring at room temperature followed by extensive washing of the support on a medium-frit sintered glass funnel with 20% ethanol in 50 m*M* $KPO_4$, 1.0 m*M* EDTA (pH 6.2) buffer.

The support was dried and transferred to a screw-capped glass bottle and 100 mL of the same buffer was added to the support. This support can be stored for at least several months in a refrigerator at +4°C without any appreciable change in amino group content. The density of the amino groups and disulfides of the support was measured by 2,4,6-trinitrobenzene sulfonic acid (TNBS) *(26)* and 2-nitro-5-thiosulfobenzonte (NTSB) *(27)* methods, respectively (see Analyses).

### *Synthesis of Reducing Support (dihydrolipoic acid–Toyopearl)*

Preparation of dihydrolipoic acid (dHLPA) immobilized on AF-Amino-650M Toyopearl support was carried out essentially as described by Gorecki and Patchornik *(28)*. Briefly, the support was synthesized by mixing 0.91 g of *N*-(lipoyloxy)succinimide (mol wt = 303.4, 3.0 mmol) dissolved in 5 mL of a dry dimethylformamide (DMF) *(28)* to 10 mL of AF-Amino-650M Toyopearl support (the density of amine groups is 100 µmol/mL of a drained support) in a 50-mL polyethylene tube. This was followed by the addition of 15 mL of DMF to the slurry. Six milliliters of 1.0 *M* $NaHCO_3$ was added to the slurry in 1.0-mL increments. The tube was sealed and incubated with constant rotation overnight at room temperature. The next day the slurry was exhaustively washed on a sintered glass filter sequentially with DMF, 50% DMF, water, and, finally, with an aqueous 20% ethanolic solution. The reduction of the immobilized thioctic acid to dHLPA was performed by incubating the support in a 150 m*M* solution of *tris*-(2-carboxyethyl)phosphine hydrochloride (TCEP) in 0.1 *M* acetate buffer (pH 4.5) for 30 min at room temperature, followed by an extensive wash with $H_2O$. The presence of traces of TCEP in the washing solution was determined by the method of Han and Han *(29)*. The reducing capacity of the support was close to 100 µmol of dHLPA/mL of drained support as measured by the DTNB assay *(30,31)*.

### *Stability of AE-S-S-Sepharose 6B*

The number of amino groups and disulfides of AE-S-S-Sepharose 6B (20% suspension) was measured both immediately after synthesis and after an incubation for 4 wk at 37°C in 0.2 *M* $KPO_4$ buffers at pH 6.0, 6.5, 7.0, 7.5, and 8.0 (see Analyses). All incubations were performed under sterile conditions.

### *Preparation of Glycation Mixtures*

Reaction mixtures were prepared by placing 30 mL of a 33% AE-S-S-Sepharose 6B suspension in 50 m*M* $KPO_4$, 1.0 m*M* EDTA (pH 6.2) contain-

ing 20% ethanol with a concentration of amino groups of 23.9 ± 1.0 μmol/mL of a drained support in a sterile 300-mL sintered glass filtration funnel. The slurry was dried by aspiration and washed three times with 75 mL of 70% ethanol (pH 6.9) under a laminar flow hood. The support was then carefully transferred into a 50-mL sterile culture flask followed by the addition of sterile isobiocytin and the sugar of choice, both dissolved in 0.2 *M* phosphate buffer, containing 1.0 m*M* EDTA, pH 7.0. The final concentrations of the support's free amino groups and isobiocytin in methylglyoxal, glyceraldehyde, erythrulose, and ribose glycation mixtures were kept the same at 8.3 μmol/mL, while sugar concentrations were 16.6 m*M*. In the case of glucose and fructose glycation mixtures, the sugar concentration was increased to 1.0 *M*. The final volume of all the glycation mixtures was 30.0 mL, except methylglyoxal, which was 300 mL. Toluene was added to every flask (50 μL) to prevent bacterial growth. The flasks were sealed and incubated with constant rotation at 37°C for specified periods of time in an incubator. A support with the concentration of amine groups of 23.9 ± 1.0 μmol/mL of gel was used throughout these studies.

### *Time-Course Incorporation Studies*

At specified intervals well-suspended 500-μL aliquots of the glycation mixtures (see preparation of glycation mixtures) were withdrawn under a laminar hood, placed in 1.5-mL cryogenic vials, and frozen in a –20°C freezer for future analyses (TNBS, NTSB, HABA-avidin *[32]*; see Analyses).

### *Preparation of Crude Fraction of Sugar-Mediated Modifications and Crosslinks*

Aliquots from the aforementioned glycation mixtures ($V$ = 24.0 mL) were removed at specified times and packed into the column. The column was washed with a degassed buffer consisting of 20% methanol, 0.1% trifluoroacetic acid (TFA), 0.1% triethylamine (pH 6.0) at a flow rate of 1.0 mL/min at room temperature until the absorbance of the effluent approached zero at λ = 220/280/330 nm. The flow was stopped and the column was filled with degassed 20% methanol, 0.1% TFA-0.1% triethylamine (TEA) buffer, containing 1.0 *M* 2-MeSH (pH 8.0) at a flow rate of 1.0 mL/min. The column was chromatographed with this buffer until the bed of the column was filled with the reducing eluent at which time the flow was stopped. A positive reaction with methanolic 10 m*M* DTNB solution was used to monitor 2-MeSH coming out of the column. Usually, yellow color developed with the first drop of the effluent containing 2-MeSH. The column was incubated for 60 min at room temperature. At the end of the incubation period, flow was started again and 3.0-mL fractions were collected. A total of 60 mL of buffer was used at a flow rate of 0.5 mL/min. Aliquots from the fractions were diluted (1:10) and absorbance at 280 and 330 nm was measured. Fractions obtained from 2-MeSH elution that had an absorbance at λ = 280 and 330 nm were collected and evaporated to dryness

with a rotary evaporator at 37°C. They were redissolved in $H_2O$ and measured for biotin concentration by the HABA:avidin assay (see Analyses). The fractions were frozen and kept at –70°C until use.

### *Purification of Crosslink Fraction from Crude Fractions of Sugar-Derived Modifications and Crosslinks*

Every sugar glycation mixture presented herein was separated on two similar monomeric avidin columns with $V$ ~ 13 to 14 mL. Before use, the columns were saturated with biotin followed by releasing biotin from "reversible" binding sites using a treatment with 0.1 *M* glycine buffer at pH 2.5. Such pretreatment left "nonexchangeable" sites blocked by tightly bound biotin. The columns were extensively washed and reequilibrated in 0.1% TFA-0.1% TEA solution, pH 6.5, before any separation.

To remove any disulfides that may have formed during the preparation of the crude fractions of AGE crosslinks, all glycation fractions were reduced with dHLPA-Toyopearl support prior to separation. A total of 250–400 µL of each sugar glycation mixture (containing approx 1.0 m*M* concentrations of biotinylated material) was used for purification on monomeric avidin columns. The glycation mixture was placed in a 15-mL polyethylene tube and its volume was adjusted to 1.0 mL with 0.1% TFA-0.1% TEA solution, pH 8.0, followed by the addition of ~1.0 mL of 90% suspension of the dHLPA-support suspension in water, and the pH of the slurry was adjusted to ~8.0 with concentrated TEA. The solution was vortexed and flushed with argon for 1 to 2 min, and the pH was readjusted to 8.0 and the tube tightly sealed. The entire mixture was incubated for 30 min with occasional vortexing. At the end of the incubation period, the slurry was transferred into AutoVial 5, and the reduced contents were filtered into a 15-mL polyethylene tube using a plunger. The dHLPA-support was additionally washed with six 2-mL aliquots of degassed 0.1% TFA-TEA buffer, pH 6.5, until the total volume of the supernatant of the glycating mixture reached approx 13.0 mL. The pH of the supernatant was adjusted to 6.5 with concentrated TFA, and the mixture was loaded onto a monomeric avidin column equilibrated with deaerated 0.1% TFA/TEA buffer, pH 6.5, at a flow rate of 2.0 mL/min until a sample entered the column bed. The flow was stopped and the column was incubated for 30 min at room temperature. At the end of the incubation period, the flow was restarted with a pump set at 2.0 mL/min. The column was eluted first with 150 mL of the same deaerated solution followed by another 60 mL of degassed $H_2O$. The bound crosslink fraction was then removed by applying deaerated eluting solution of 0.1% TFA-TEA solution at pH 2.25 at 2 mL/min and collecting 6-mL fractions (150 mL total). At the end of the separation, the collected fractions were dried using a Savant rotary evaporator. The dried fractions were dissolved in 1.0 mL of degassed $H_2O$ and the absorbance at 220 and 330 nm was measured. Aliquots of the glycating mixture as well as aliquots of the bound fraction were tested for the SH-group and biotin content by DTNB and HABA assays correspondingly.

## Analyses

### Quantification of Amino Groups of AE-S-S-Sepharose 6B

A modification of the assay of Snyder and Sobocinski *(27)* was used to quantify the free amino groups of the gel. Briefly, aliquots of a well-suspended slurry were placed into syringeless filter devices (AutoVial 5) and washed four times with 4.0 mL of 1.0 *M* NaCl, pH 6.2. The device's outlet was capped and 2.0 mL of 0.1 *M* borate buffer, pH 9.3, was added to each device along with the addition of 100 µL of 0.05 *M* TNBS with vigorous stirring. The assay mixtures were incubated for 20 min with occasional vortexing. At the end of the incubation period, the assay solutions were filtered and the modified Sepharose support was washed four times with 4.0 mL of 50 m*M* $KPO_4$, 1.0 m*M* DTPA (pH 6.2). The outlets of the AutoVial 5 devices were sealed again, and 2.0 mL of a freshly made 1% solution of DTT in 0.1 *M* borate buffer (pH 9.3) was added with vigorous stirring of the filter devices. The assay mixtures were incubated for another 20 min with occasional vortexing. At the end of the incubation period, the assay mixtures were filtered into separate tubes, and their absorbance was measured against 0.1 *M* borate buffer (pH 9.3) at $\lambda$ = 420 nm. The concentration of amino groups on AE-S-S-Sepharose 6B support was derived by comparing the values obtained by this assay with a standard curve prepared from assaying a standard mixture of amino acids.

### Measurement of Thiol Groups

The thiol concentration of the preparations used was spectrophotometrically determined by the titration of sulfhydryls with DTNB as described by Ellman *(30)* and using $\varepsilon_{412}$ = 14,150 $M^{-1}$/cm *(32)*.

### Quantification of Disulfides of AE-S-S-Sepharose 6B by NTSB Assay

NTSB was synthesized from DTNB by the method of Thannhauser et al. *(26)* with few modifications. In our hands, the total oxidation of DTNB (>99%) to NTSB by sodium sulfite in the presence of oxygen was complete within 3 h. The NTSB assay solution was prepared exactly as suggested by Thannhauser et al. *(26)*. To detect the disulfides, either in a newly synthesized or glycated AE-$S_2$-Sepharose 6B, an aliquot of the particular well-suspended slurry was pipetted onto the filter bed of an AutoVial 5 and washed four times with 4.0 mL of 50 m*M* $KPO_4$, 1.0 m*M* EDTA (pH 6.2). The outlet of the device was capped and 3.0 mL of NTSB assay solution was added to it with careful vortexing. The reaction mixture was incubated for 10 min and the supernatant was removed by filtration at the end of the incubation period. The absorbance of the sample at 412 nm was recorded against a blank of 3.0 mL of NTSB assay solution and the appropriate amount of water. The concentration of the disulfides was calculated based on the 2-nitro-5-thiobenzoic acid's extinction coefficient (14,150 $M^{-1}$/cm at $\lambda$ = 412 nm) *(32)* and expressed as moles/milliliter of a drained gel. All experiments were performed in triplicate.

### AE-S-S-Sepharose 6B Incorporation Studies

For quantitation of sugar-dependent incorporation of isobiocytin into AE-$S_2$-Sepharose 6B, the HABA:avidin assay was used *(31)* with some modifications. Briefly, an aliquot of the well-suspended slurry glycated for a specified period was placed into a syringeless filter device and washed four times with 4.0 mL of 50 m*M* $KPO_4$, 0.15 *M* NaCl (pH 6.0). The device outlet was capped and 1.0 mL of 20 m*M* TCEP in the same buffer was added to the device with vortexing. The mixture was incubated for 20 min and the supernatant was collected into a separate tube. An aliquot of supernatant was mixed with the HABA:avidin reagent, allowed to incubate for 10 min, and read against a blank at $\lambda = 500$ nm. The concentration of the crosslinks formed between AE-S-S-Sepharose and isobiocytin was calculated by using $\varepsilon_{500} = 35{,}500\ M^{-1}/\text{cm}$ of HABA:avidin complex *(31)*. This assay mixture tolerates a presence of 150 µL of 20 m*M* TCEP without any adverse effects for at least 2 to 3 h of incubation.

### Measurement of Crosslinks by HABA:Avidin Assay

The crosslink concentration of the preparations presented herein was spectrophotometrically determined at $\lambda = 500$ nm by using the Haba:avidin assay *(31)*. All calculations of the crosslink concentration are derived from using $\varepsilon_{500} = 35{,}500\ M^{-1}/\text{cm}$ *(31)*.

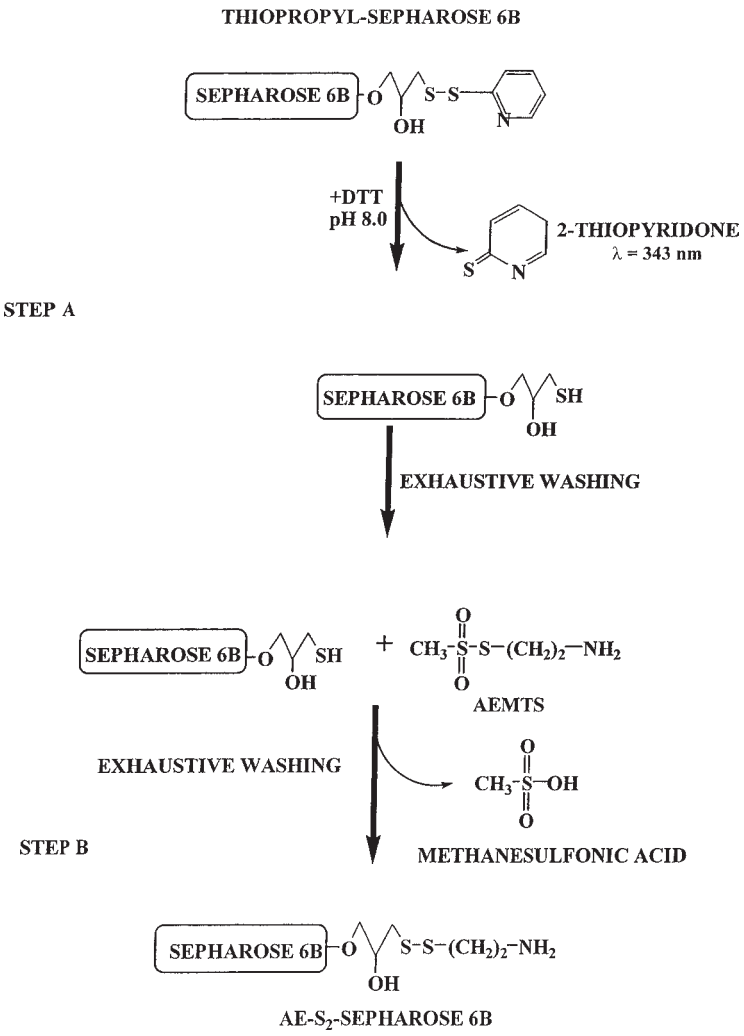
### Additional Methods

Fluorescence spectra of the crude preparations and fractions obtained after separation over the monomeric avidin column were recorded using a Hitachi model F-2500 spectrofluorometer. All the spectrophotomeric work was performed on a Genesys 5, Milton Roy, or Varian UV/VIS model Carry 1E spectrophotometer.

## Results

### *Conditions for Synthesis of 2-Aminoethane Disulfide Sepharose 6B*

The 2-aminoethane disulfide Sepharose 6B support was synthesized by a two-step procedure: reduction of the original thiopropyl Sepharose 6B with DTT at basic pH followed by an extensive washing (Scheme 1) and modification of the available sulfhydryls with 2-AE-MTS (Scheme 1, step B). The first step, the reduction of the original 2-thiopyridonyl disulfides of the support to free SH-groups, was carried out at pH 8.35 for 1 h at room temperature in the presence of a fourfold excess of DTT (Scheme 1, step A). The inset in Fig. 1 shows that the formation of the sulfhydryl groups on the support owing to the treatment with DTT is fast and that the reaction was completed within the first 15 min of incubation. The concentration of SH-groups after 1 h of incubation was 37.7 ± 0.26 µmol/mL of a drained support as determined by the DTT-dependent release of 2-thiopyridone from the support. These data were verified by measuring the concentration of SH-groups of the support by the DTNB assay. This assay showed that the




Scheme 1. Steps in preparation of 2-aminoethane disulfide Sepharose 6B.

concentration of free SH-groups on the gel available for the reaction with DTNB after exhaustive washing of the support was 37.05 ± 1.67 μmol/mL of a drained support (see inset in Fig. 1; numbers in parentheses). These data are in good agreement with the data on characteristics of the support provided by the manufacturer (18–31 μmol/mL of a drained support).

Immobilization of the cysteamine residues on the support was performed by reacting of the free sulfhydryl groups of the reduced support with 2-AE-MTS at pH 8.35 for 1 h at room temperature (see Scheme 1, step B and Fig. 1). This particular reaction is quantitative, and disulfide formation went to completion within 60 min to almost 100% yield. Figure 1 shows that the support contained almost no SH-groups after 1 h of reaction, and the concentration of immobilized cysteamine was about 43 μmol/mL

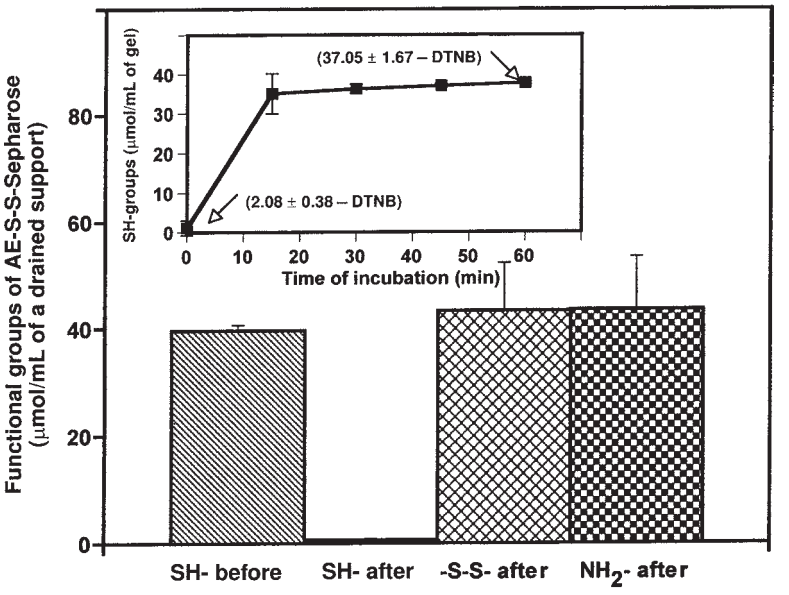



Fig. 1. Introduction of amino groups into thiopropyl Sepharose 6B by a treatment with 2-aminoethylmethanethiosulfonate after 1 h of incubation at room temperature. The insert on the graph shows the reduction of thiopropyl Sepharose 6B by DTT at pH 8.3. The points on the curve show the data obtained by measuring an absorbance of 2-thiopyridone released by DTT treatment into the supernatant taken at $\lambda = 343$ nm at the intervals shown in the graph. Numbers in parentheses represent the DTNB assay data of concentration of SH-groups of the support before and after DTT treatment. All the data show the average results of three independent preparations performed in triplicate.

of a drained gel, as can be seen from the data obtained by disulfide and amino group assays (see Fig. 1).

## *Stability of 2-Aminoethane Disulfide Sepharose 6B*

The stability of the 2-aminoethane disulfide Sepharose 6B as a function of pH is shown in Fig. 2. When incubated for 1 mo at 37°C under sterile conditions, there was a 12–15% loss of the total amino groups of the support with increasing slurry pH. The data collected from the disulfide and amino group assays, however, showed that almost 100% disulfides remained at all pH values. The higher concentration of disulfides measured argues that the disulfide assay detects the total disulfide content of the support, which includes disulfides that were formed on 2-aminoethane disulfide Sepharose 6B owing to mixed disulfide exchange during an incubation and cysteamine disulfides Sepharose 6B themselves. In addition, the support was extremely stable in 20% ethanolic solutions at pH close to 6.0, showing no significant decrease in amino group content after 6 mo of storage at +4°C (data not shown).

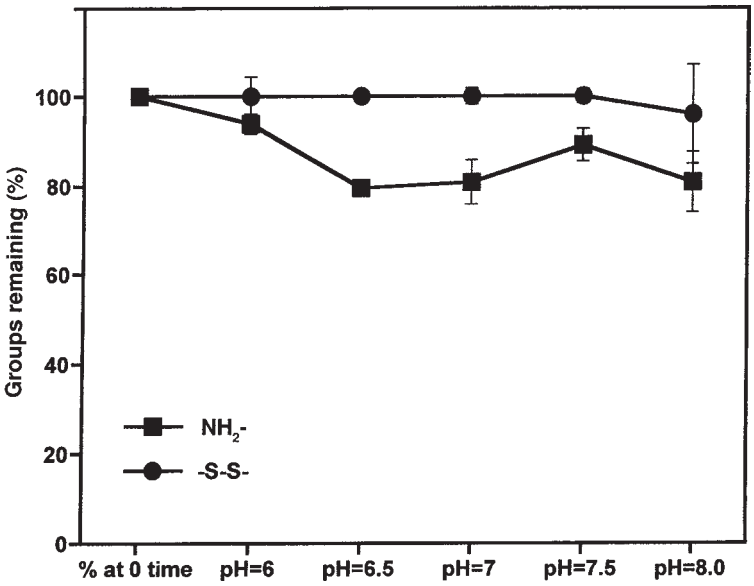



Fig. 2. Stability of AE-S-S-Sepharose 6B after 1 mo of incubation at 37°C at different pH values and under sterile conditions. The upper curve shows the data obtained from NTSB-disulfide assay, and the lower curve represents the results of TNBS-free amino group assay of the support. All measurements were performed in triplicate.

## *Glycation of AE-S-S-Sepharose 6B with Different Sugars*

α-Biotinylated lysine was incubated with cysteamine-disulfide Sepharose in the presence of glucose, fructose, ribose, erythrulose, glyceraldehyde, and methylglyoxal. All experiments were performed in 0.2 *M* phosphate buffer under conditions that favor the formation of the sugar-mediated crosslinks as described by Neglia et al. *(33)*, Eble et al. *(34)*, Watkins et al. *(35)*, and Lee et al. *(36)*. In the case of glucose, fructose, and ribose we used unchelated buffers, and for the rest of the sugars a 1.0 m*M* concentration of EDTA was present in the buffers throughout the experiments (see Materials and Methods). The ratio of the immobilized amine: sugar:isobiocytin was 1:2:1 except for the glycation mixtures that contained glucose and fructose, where it was 1:120:1.

Figure 3 shows measurements of the functional groups on 2-AE-S-S-Sepharose 6B glycated after specified periods in the presence of different sugars. As can be seen from Fig. 3A, all sugars caused substantial loss of the detectable gel-conjugated amino groups (75–93%). Consistent with the previous results on the protein glycation with the same sugars *(36)*, short-chain sugars were the most reactive and glycated 19–22 of 24 μmol/mL of original amino groups during the incubations. Interestingly, ribose showed relatively high modifying activity and accounted for the loss of 18.0 ± 1.4 μmol of the amino groups/mL on the support after 2 wk of incubation. The least reactive sugars were glucose and fructose. At 1.0 *M* concentrations, these carbohydrates required 1 mo of incubation to cause the loss of

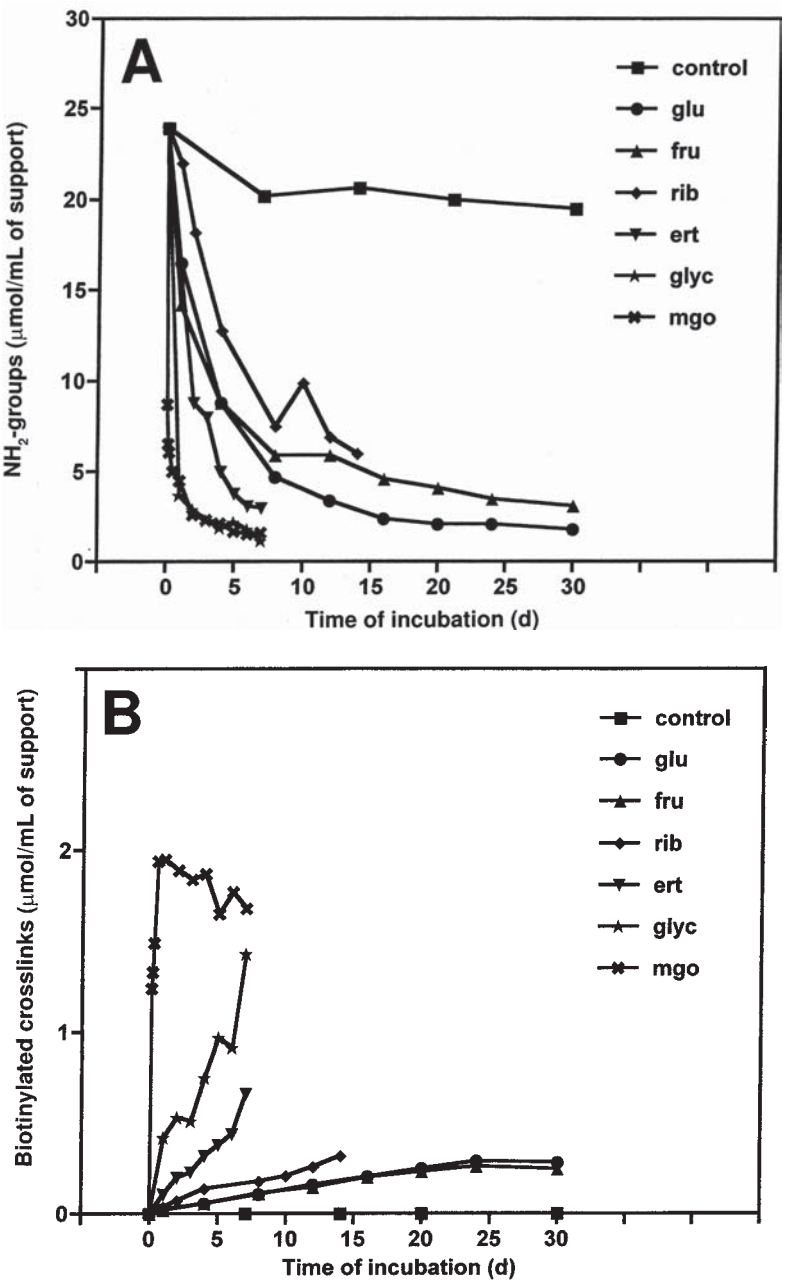



Fig. 3. Concentration of free amino groups **(A)**, incorporated isobiocytin **(B)**, and disulfides **(C)** of AE-S-S-Sepharose 6B during incubation of the support with different sugars at 37°C under sterile conditions for the duration from 1 wk to 1 mo (see Materials and Methods for details). The final concentrations of the support's free amino groups and isobiocytin in methylglyoxal, glyceraldehyde, erythrulose, and ribose glycation mixtures were kept the same at 8.3 μmol/mL, while sugar concentrations were 16.6 m*M*. In the case of glucose and fructose glycation mixtures, the sugar concentration was increased to 1.0 *M*. All assays were performed in duplicate, except the NTSB-disulfide assays, which were performed in triplicate.

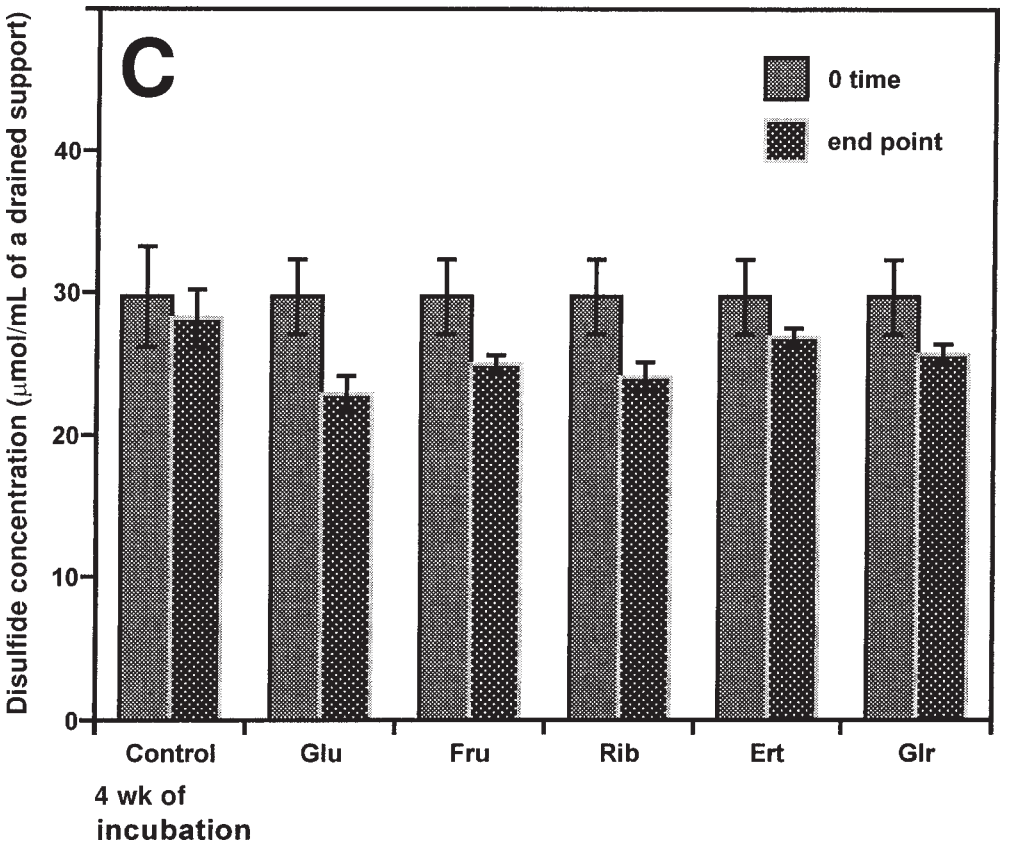



Fig. 3. *(continued)*

amino groups comparable with that seen in the methylglyoxal glycation mixtures (~90%).

The use of α-biotinylated Lys in these studies allowed us to detect the relative crosslink-synthesizing activity of the sugars and to estimate the relative yields of the sugar-specific crosslinks from the glycated supports. Figure 3B shows the sugar-mediated incorporation of isobiocytin into the support. The data represent HABA:avidin assays performed on the biotinylated fractions released from the modified gel by a short-term treatment with TCEP. Methylglyoxal had the highest crosslinking activity and accounted for 7.8–8.2% of all the modified amino groups of the support being crosslinked to isobiocytin, reaching a density of 1.68 ± 0.2 μmol of biotinylated crosslinks/mL of support. With an increase in the length of the carbon chain, the crosslinking ability of the sugars gradually diminished, reaching 5.8, 2.8, and 1.3% of the support total amino groups for glyceraldehyde, erythrulose, and ribose, respectively (see Table 1). Glucose and fructose at a concentration of 1.0 *M* had the lowest crosslinking ability, amounting only to approx 1% of total amino groups.

Table 1
Yields of Sugar-Mediated Crosslinks Released from Glycated Supports[a] with 1.0 *M* 2-MeSH

| Glycation mixture | Concentration of crosslinks/mL of support ($\times 10^{-6}$ *M*) as assayed by HABA:avidin method | Volume of support used (mL) | Theoretical yield ($\times 10^{-6}$ mol) | Amount of crosslinks recovered ($\times 10^{-6}$ mol) | Total yield[b] (%) |
|---|---|---|---|---|---|
| Methylglyoxal | 1.68 ± 0.14 | 8.3 | 13.97 | 9.56 ± 0.08[c] | 56.8[c] |
| Glyceraldehyde | 1.43 ± 0.02 | 8.3 | 11.87 | 9.55 ± 0.01 | 80.5 |
| Erythrulose | 0.66 ± 0.04 | 8.3 | 5.48 | 6.04 ± 0.02 | 110.3 |
| Ribose | 0.32 ± 0.02 | 8.3 | 2.66 | 4.17 ± 0.04 | 157.0 |
| Fructose | 0.25 ± 0.02 | 8.3 | 2.06 | 2.38 ± 0.01 | 115.6 |
| Glucose | 0.28 ± 0.01 | 8.3 | 2.36 | 2.49 ± 0.02 | 105.0 |

[a]The density of the amino groups of the unmodified AE-S-S-Sepharose was 23.9 ± 1.0 µmol/mL of a drained support.
[b]The yields shown represent the results of HABA:avidin assay of the crude fractions after they had been dried on a rotary evaporator and redissolved in 0.1% TEA:TFA, pH 6.5.
[c]Part of this fraction was lost during drying on a rotary evaporator.

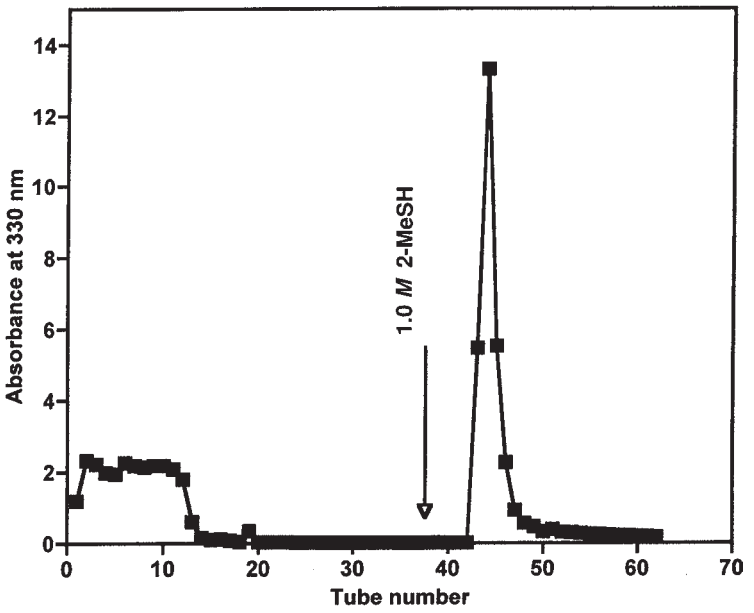



Fig. 4. Purification of the crude fraction of glyceraldehyde AGE modifications and biotinylated crosslinks from unbound material. The separation of the bound and unbound material was conducted at flow rates of 1 mL/min.

Determination of the density of the support's disulfide groups remaining after glycation reactions revealed that only 12–15% of disulfides were lost during the incubation with the sugars (see Fig. 3C). Since almost 90% of the support's disulfides were left intact after the incubations up to 1 mo in the presence of the sugars, one can argue that the disulfide bond of the support did not participate significantly in amine-carbonyl condensation reactions.

### *Purification of Crude Fractions of Sugar-Mediated Modifications and Crosslinks*

To isolate the fraction of AGE support-bound products, unbound reactants were removed by extensive washing and the glycated 2-aminoethylthiol groups were detached with 1.0 *M* 2-MeSH. As can be seen in Fig. 4, the unbound material was completely removed from the column in the first 45–50 mL of the effluent judged by the absorbance readings taken at 220 and 330 nm. Treatment of the column with 1.0 *M* 2-MeSH at pH 8.0 released almost all the bound material in the first 20 mL after the elution had begun. Table 2 shows that the recoveries of the biotinylated material were in good agreement with the results obtained from the data on the sugar-mediated incorporation of isobiocytin into AE-S-S-Sepharose 6B (see Fig. 3B). Only glyceraldehyde showed somewhat lower yields (80%), which may reflect poor handling of the material during the evaporation process. Taking into account the data presented in Table 1, the corrected

Table 2
Yields of Biotinylated Crosslinks After Separation of Crude Fractions over Monomeric Avidin Column

| Glycation mixture | Original concentration of crosslinks (m*M*) | Amount of crosslinks loaded (nmol) | Amount of crosslinks eluted in bound fraction (nmol)[a] | Amount of crosslinks eluted in unbound fraction (nmol)[a] | Yield of column-bound crosslinks (%) |
|---|---|---|---|---|---|
| Methylglyoxal | 0.89 ± 0.02 | 358 | 277 ± 8 | 129 ± 28 | 77.4 |
| Glyceraldehyde | 1.64 ± 0.04 | 410 | 247 ± 33 | 163 ± 15 | 60.2 |
| Erythrulose | 1.54 ± 0.14 | 389 | 194 ± 71 | 199 ± 16 | 50.0 |
| Ribose | 1.32 ± 0.36 | 330 | 176 ± 24 | ND[b] | 53.3 |
| Fructose | 1.06 ± 0.03 | 265 | 156 ± 16 | 113 ± 22 | 58.9 |
| Glucose | 0.83 ± 0.02 | 208 | 142 ± 8 | 111 ± 11 | 68.3 |

[a]The results were calculated based on the data accumulated from the HABA:avidin assays and represent the average of two separations performed on two separate columns. All the HABA:avidin assays were performed in triplicate.
[b]ND, not determined.

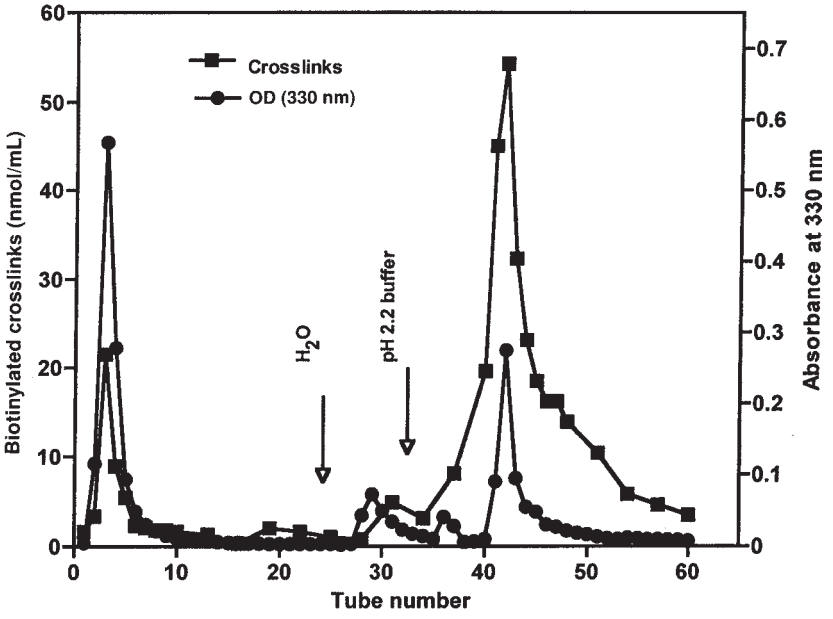



Fig. 5. A typical chromatographic profile of purification of the fraction of biotinylated crosslinks from the crude preparation of methylglyoxal-derived AGEs over the monomeric avidin column. The profile represents an average result derived from the data accumulated from the two independent separations (see Materials and Methods and Results for details).

numbers for the moles of isobiocytin incorporated per milliliter of a drained AE-S-S-Sepharose 6B by glucose, fructose, ribose, and erythrulose are 0.3 (1.25%), 0.287 (1.2%), 0.502 (2.1%), and 0.728 (3.1%) μmol/mL of support, respectively.

### *Purification of Fraction of Isobiocytin Containing Crosslinks*

To eliminate possible contamination of the biotinylated fraction by modifications, 2-MeSH, and cysteamine that could be introduced by the formation of mixed disulfides with the crosslinks, we reduced the crude bound glycation mixtures by treatment with dHLPA-reducing gel support at pH 8.0. The pH of the filtered reduced fractions was then readjusted to 6.5 and applied onto a monomeric avidin column ($V \approx 13$ to 14 mL). Since the binding capacity of monomeric avidin support is relatively low (12–19 nmol of biotin/mL of a drained support according to the manufacturer), the columns were loaded with the amounts of the biotinylated material that exceeded a theoretical binding capacity of the column in order to obtain the highest possible yields of the pure biotinylated crosslinks.

Figure 5 represents a typical separation of the reduced crude methylglyoxal bound fraction from the previous separation over the monomeric avidin column (see Materials and Methods). The separation shows that the column was able to resolve bound and unbound fractions completely and

collect the bound crosslinks in a pure state. All of the nonbiotinylated material and some of the biotinylated crosslinks eluted very early during the separation ($V \sim 60$ mL). The bound fraction was released from the column once the pH of the eluting buffer was changed to 2.2, liberating most of the pure bound material in 150 mL of the effluent. We also performed several experiments in which the biotinylated crosslinks were eluted from the column with a buffer that contained 2.0 m*M* biotin at pH 6.5 (data not shown). This procedure produced chromatographic profiles identical to the ones observed for the low pH profiles as judged by an absorbance at 330 nm. These data confirm the presence of the biotinylated substances in these fractions *(37,38)*. Table 2 shows the yields of the biotinylated crosslinks obtained after various crude fractions were separated over the monomeric avidin column. The amounts of the purified crosslink in fractions eluted with a low pH buffer were in good agreement with the aforementioned theoretical capacities of the support provided by the manufacturer. Since the sum of the crosslinks in the bound and unbound fractions was close to 100% compared to the amounts of the loaded material for all the fractions, these results confirm the data on the sugar-mediated incorporation of isobiocytin into AE-S-S-Sepharose (see Table 2).

### *Spectral Characteristics of Biotinylated Crosslinks*

The absorbance and fluorescence spectra of biotinylated crosslinks at the end points of glycation are shown in Fig. 6. There is a little difference in the shape of the various spectra. All the absorbance spectral curves exhibited diffuse shoulders in the regions of 280–290 and 320–350 nm ($\lambda_{max}$ close to 330 nm). Such a pattern usually appears when amino acids are being glycated with lower sugars or degradation products of ascorbic acid *(13,14)*. Since the isolated crosslink fractions from the different glycation mixtures were in the relatively pure state, we calculated average extinction coefficients of the crosslinks at $\lambda_{max} = 330$ nm. They are 2850, 5100, 6300, 2100, 5900, and 3000 $M^{-1}$/cm for glucose, fructose, ribose, erythrulose, glyceraldehyde, and methylglyoxal, respectively.

Fluorophore formation is usually associated with the formation of advanced glycated end products *(15,39–41)* and has been reported to be associated with AGE formation in diabetes-related and aged and cataractous proteins *(16,17)*. In our experiments, all the purified crude fractions of biotinylated crosslinks were fluorescent to some extent when they were excited at $\lambda_{max} = 330$ nm. All sugar glycation mixtures containing four to six carbon sugars exhibited emission maxima in the 405- to 415-nm region. The ribose crosslink fraction exhibited the highest fluorescence, probably rendering to the formation of the structures similar to vesperlysines *(7,8,11)* during the progression of the glycation reaction. The emission maximums of glyceraldehyde and methylglyoxal crosslinks are red shifted and showed $\lambda_{max}$ in the region of 430–440 nm. The glyceraldehyde and methylglyoxal crosslinks were also among the lowest fluorescing compounds. This is probably owing to formation of adducts structurally similar to MOLD,

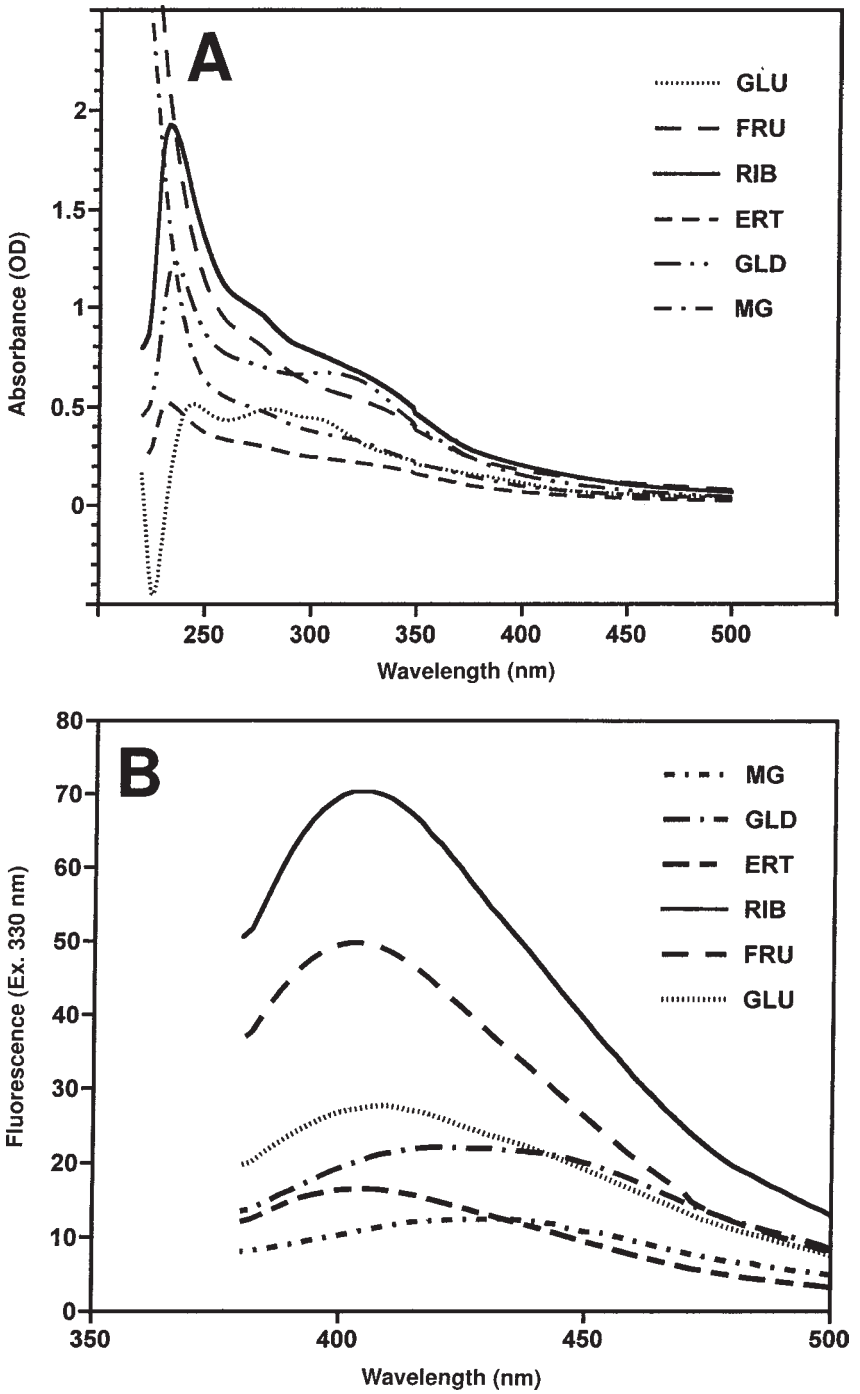



Fig. 6. Absorbance **(A)** and fluorescence **(B)** spectra of the purified sugar-mediated biotinylated crosslinks. The emission spectra were recorded using an excitation wavelength of 330 nm. The concentration of the crosslinks used for the absorption spectra was $1 \times 10^{-4}$ *M*, and the concentration of the crosslinks used for fluorescence spectra was $1 \times 10^{-5}$ *M*.

which is the major product of the reaction between Lys and methylglyoxal. It is known that this compound is a poor chromophore and does not possess any fluorescence *(9,10)*.

## Discussion

To date, only a few general methods have been developed for isolation of AGE crosslinks from proteins extracted from pathologic tissues or from artificial glycation mixtures *(42,43)*. The isolation of glycation-mediated protein-protein crosslinks is extremely difficult because exhaustive proteolytic cleavage often does not proceed to the amino acid stage and produces a collection of different peptides of different composition, which seriously hampers their structural characterization *(44,45)*. If acid hydrolysis is used instead of proteolysis, it may lead to the destruction of the AGE crosslinks, because most AGE crosslinks have been reported as acid labile *(20)*. While acid-stable crosslinks have been isolated *(6–11,16,18–20)*, they likely represent a small fraction of the total crosslinks present *(20)*. The isolation of the crosslinks from the artificial glycation mixtures containing $N^{\alpha}$-blocked Lys or Arg and sugars is complicated by the presence of AGE modifications and complex sugar condensation products from which crosslinks cannot be easily separated and identified *(8–11)*. Except for MOLD and GOLD, most of the AGE crosslinks isolated and characterized so far are fluorescent compounds (pentosidine, vesperlysine A, threosidine, and furopyrrolopyridine), a property that greatly facilitated their purification and characterization *(6–8,11,18–20,43)*. On the other hand, most of the glucose AGEs are nonfluorescent *(20)* and probably do not contain highly ultraviolet (UV)-absorbing chromophores in their structure (e.g., MOLD or GOLD) *(9,10,16)*.

To identify the chemical nature of glycation-mediated crosslinks formed in vitro, we developed a method and demonstrated, for the first time, the practical usefulness of AE-S-S-Sepharose 6B and isobiocytin for the preparation and purification of total AGE-Lys-Lys-like crosslinks. The system employs the sugar-dependent crosslinking of $N^{\alpha}$-biotinylated Lys into the cysteamine modified-agarose support. This allows the separation of unbound material in the glycation mixture from agarose-bound material, and since the bond between cysteamine and thiopropyl-Sepharose 6B is a disulfide bond, it is possible to release agarose-bound modifications by a treatment with 2-mercaptoethanol without the need for acid hydrolysis. The support withstands incubations at physiologic pH for periods up to 1 mo at 37°C, which is in good agreement with the results of Batista-Viera et al. *(46)* on the stability of this type of support at different pH values. Since the concentration of the disulfides between cysteamine and Sepharose 6B did not change during incubation with any of the sugars used, this rules out any significant involvement of disulfides in the support in the Maillard reactions (cf. Fig. 2 and Fig. 3C).

Although we observed an extensive loss of measurable amino groups on the support in the presence of all the sugars used, methylglyoxal was the

most reactive and caused almost 90% loss of the amino groups of support within 24 h of incubation. Glucose and fructose reached such a degree of modification only after 2 to 3 wk of incubation at a concentration of 1.0 *M* (see Fig. 3A), because the glycation reaction between amino groups and reducing sugars is determined by the extent of their open chain reactive form *(47)*. The small percentage of glucose (0.002%) in the open chain reactive form correlated with the reduced ability of this sugar to modify the amino groups of the support, whereas the short carbon-sugars (2C-4C), erythrulose, glyceraldehyde, and methylglyoxal, which mainly occur in the open chain form, demonstrated much higher modification ability at lower concentration and shorter incubation periods *(36)*. Most important, the kinetics of glycation and loss of the amino groups of the support in the presence of glucose and methylglyoxal are very close to the results on glycation of Lys residues in the structure of RNase A and lysozyme by the same sugars reported by Watkins et al. *(35)*, Eble et al. *(34)*, and Neglia et al. *(33)*. Since our experiments were performed under similar experimental conditions and duration, this argues that the amino groups of attached cysteamine behaved similarly to ε-amino groups of lysine in the structure of Rnase A and lysozyme *(33–35)*.

The reactivity of the sugars for crosslinking was similar to their ability to glycate the support's amino groups (see Fig. 3A,B). This is not unexpected because the glycation reaction (formation of Shiff's bases and Amadori compounds) must proceed before crosslinking can be demonstrated *(34,36,48)*. We observed that glucose and fructose showed equally poor crosslinking ability (about 1% of all amino group sugar-dependent modifications) even in the presence of a 1.0 *M* concentration of sugars for 1 mo of incubation, whereas short-chain sugars, erythrulose (3.1%), glyceraldehyde (5.8%), and methylglyoxal (8%), were all many times more reactive (see Fig. 3B). Since the relative incorporation of isobiocytin into the support by the sugars used is in good agreement with the data on the ribose and glucose-dependent incorporation of [$^{14}$C] Lys in poly-L-lysine *(48)*, as well as all [$^{14}$C] $N^{\alpha}$-formyllysine by the sugar used herein in soluble lens proteins published from our laboratory *(36)*, it can be argued that similar mechanisms are involved in sugar-dependent incorporation of Lys in the proteins and Lys in the structure of isobiocytin into cysteaminyl-Sepharose 6B.

The biotin-avidin system, which has been productively exploited in other contexts, can be applied to a separation of glycation crosslinks synthesized in vitro. Provided that crosslinks in our preparations (Fig. 5) have an attached biotin, it was possible to separate them in pure form from the rest of the glycation mixture by affinity chromatography on a monomeric avidin column (see Fig. 5 and Table 2). The presence of the SH-groups in the structure of the crosslinks, though, required additional precautions to be taken in the handling and preparation of the crosslinks for separations. It is known that SH-groups are prone to the formation of mixed disulfides even at neutral pH values *(49)*. The crosslinks in the crude prepa-

rations eluted from the glycated cysteaminyl-Sepharose 6B represent only a small fraction (at most 8% in the case of methylglyoxal) among all other AGE modifications and cysteamine present in these fractions. This means that on storage or purification there is a possibility that crosslinks can form disulfides either among themselves or, most important, with cysteamine and AGE modifications. To prevent this possible source of contamination in the crosslink fractions, the total fraction of AGEs was reduced before its loading onto a monomeric avidin column with the dHLPA reducing support. If contamination of crosslinks and AGE modification with a reducing agent is not of concern, an alternative approach that uses TCEP as a reducing agent in all the buffers applied for the monoavidin separations would circumvent such a contamination of the crosslinks in the future. This is because TCEP reduces disulfides at a pH lower than neutral, requires a much lower ratio of TCEP:disulfide than, e.g., DTT or 2-mercaptoethanol and will keep all the compounds in the crude preparation in a reduced state during purification *(29)*. The contamination-free AGE crosslinks were eluted from the monoavidin columns by using the buffer with pH 2.2–2.5. The reason for using such elution is simple. The elution profiles of the biotinylated crosslinks using 2.0 m*M* biotin at neutral pH instead of low pH buffers were identical to the one shown in Fig. 5, as judged from the absorbance of the eluted fractions taken at 330 nm (data not shown). Yet, the presence of biotin in the buffer for eluting the column-bound crosslinks made it impossible to separate the crosslinks from biotin after purification owing to the low solubility of biotin in water *(50)* and prevented further characterization of these fractions.

The recoveries of the biotinylated AGE Lys-Lys-like crosslinks generated by each sugar after their purification over a monomeric avidin column were in good agreement with the predicted theoretical yields obtained from determining the sugar-dependent isobiocytin incorporation into glycated supports (see Tables 1 and 2). Depending on the sugar used, all the biotinylated fractions showed recoveries of 60–75% from theoretical. This also demonstrates that even using 8–10 mL of support, one can generate micromolar quantities of crosslinks in amounts sufficient for further structural characterization. For example, this quantity of the gel required only a 7-d incubation in the presence of isobiocytin and methylglyoxal and already produced approx 6 mg of structurally unaltered Lys-Lys-like crosslinks, considering that an average molecular weight of these adducts is about 0.6 kD. In this regard, scaling up the reaction mixtures as well as using a monomeric avidin support with biotin binding capacity higher than UltraLink monomeric avidin columns used in this study (higher then 12–19 nmol of biotin/mL of support) would make it possible to synthesize tens of milligrams of the crosslinks by using, practically, any sugar.

Although no attempts were made to purify the separate compounds from the total preparations of Lys-Lys-like crosslinks, the intrinsic presence of an SH-group in their structure would definitively facilitate their isolation. As one of the approaches to separate the particular compounds

would be a derivatization of the biotinylated crosslinks with DTNB followed by a separation of the derivatized crosslinks by reverse-phase high-performance liquid chromatography *(51)*. A similar approach has already been used for the separation of biologically active thiols and has proven to be successful in the complete separation of seven structurally related compounds, all of them non-UV absorbing *(51)*.

The other utilization of the crosslinks prepared by this method would be in the preparation of antibodies against the AGE crosslinks. It is known that most of the antibodies raised against in vitro glycated proteins recognize carboxymethyllysine as the major epitope, which is not a crosslink and may probably be a protective modification, diverting Amadori precursors from forming crosslinks *(52,53)*. This is probably owing to the very low abundance of the crosslinks even in the in vitro glycated proteins used as antigens *(36,48)*. By conjugating the SH-groups of the crosslinks prepared by this method to a macromolecular carrier through maleimide-containing heterobifunctional crosslinkers, one could prepare an immunogen with a high crosslinks:carrier ratio *(54)* and could immunize animals with the haptens that are only the crosslinks and may produce high-titer antibodies in hosts against only the AGE crosslinks *(55,56)*.

## Acknowledgments

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