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Diabetic cataract formation: Potential role of glycosylation of lens crystallins

(nonenzymatic glycosylation/sulfhydryl oxidation)

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ABSTRACT **A high glucose concentration *in vivo* or an increased glucose or glucose 6-phosphate concentration *in vitro* has been found to lead to the glycosylation of ε-amino groups of lysine residues in bovine and rat lens crystallins. *In vitro*, this glycosylation imparts an increased susceptibility of the crystallins to sulfhydryl oxidation. Disulfide crosslinks result in the formation of high molecular weight aggregates and an opalescence in the crystallin solutions. The addition of reducing agents prevents as well as reverses the formation of high molecular weight aggregates and the opalescence of the crystallins. These phenomena suggest a new interpretation of previous results on cataract formation and a new approach for development of drugs to prevent cataracts.**

The secondary complications of diabetes mellitus now account for most of the morbidity and mortality associated with this disease (1). These sequelae which affect most major organs (e.g., kidney, peripheral nerve, and eye) are of unknown etiology, although many workers believe that they may be due to an increased amount of glycoproteins in capillary basement membranes (2, 3). Several years ago it was proposed (4) that the glycosylation of hemoglobin to form hemoglobin A_{Ic} may serve as a biochemical model for the glycosylation purported to occur in diabetes (5). It was found that the glycosylation of hemoglobin A_{Ic} occurred as a postsynthetic modification throughout the life of the erythrocyte and that the rate of glycosylation was 2.7 times faster when the erythrocytes circulated in a diabetic mouse than in a normal mouse. Subsequent work has shown this modification to be 1-amino-1-deoxyfructose which is specifically attached to the amino-terminal valine in the β-chain (6, 7). This addition occurs nonenzymatically as either the reaction of glucose directly (8) or of glucose 6-phosphate (9, 10) which is then dephosphorylated. It is not clear which is predominant *in vivo*. The mechanism proposed for both is formation of a Schiff base with the amino group of valine and a subsequent Amadori rearrangement to form a fairly stable product that is reducible with sodium borohydride.

Reasoning that a similar type of glycosylation might be occurring in other tissues, we initiated a study of the ocular lens. The lens, like the erythrocyte, is not dependent on insulin for glucose uptake and hence the intracellular glucose concentration reflects the extracellular milieu (11). In the present communication we report that the crystallin proteins of the lens may be glycosylated both *in vivo* and *in vitro* at the ε-amino groups of lysine residues.

MATERIALS AND METHODS

Chemicals. D[*U*-^{14}C]glucose and D[*1*-^{14}C]glucose 6-phosphate were obtained from the Radiochemical Center, Amersham, England. Sodium[^{3}H]-borohydride was obtained from New England Nuclear, Boston, MA. Dithioerythritol, glutathione, sodium borohydride, alloxan, and [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bistris) were obtained from Sigma Chemical Co., St. Louis, MO. Medium 199 and fetal calf serum were supplied by GIBCO, Grand Island, NY. N^6-1-(1-Deoxyglucitolyl)-N^2-*t*-butoxycarbonyllysine was prepared and generously provided by G. Wilson.

Animals. Sprague–Dawley rats (150–200 g) were obtained from Taconic Farms, Germantown, NY. Diabetes was induced in the rats by an intravenous injection of a freshly prepared solution of alloxan (40 mg/kg) in 0.01 M phosphate buffer, pH 3.5 (12). Plasma glucose was measured with a glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, OH). Cataracts appeared 8–10 weeks after the onset of diabetes.

Lens Cultures. Lenses from rats were cultured on wire grids in medium 199 containing 5% fetal calf serum and 30 mM glucose according to the method of Obazawa *et al.* (13). The medium was replaced daily. After 7 and 11 days in culture, the crystallins were extracted from the whole lens in 1 ml of buffer as described below. Freshly isolated normal rat lenses served as uncultured controls.

Preparation of Crystallins. Bovine lenses were used for experiments involving isolated crystallin solutions. Eyes from freshly killed cattle were obtained from a local abbatoir. The lenses were removed on ice and all subsequent steps were performed at 1°–4°. The cortical and nuclear regions of the lenses were separated by teasing the outer two-thirds of the lens from the dense nuclear mass with scalpels. The lens tissue was then homogenized with a ground-glass tissue homogenizer in 5 mM potassium phosphate buffer (pH 6.8) under 100% nitrogen in a glove bag (I^2R Co., Cheltenham, PA). The resulting suspensions were centrifuged under nitrogen at 6500 × *g* for 1 hr and the supernatant solution was dialyzed for 24 hr against 0.05 M potassium phosphate buffer (pH 7.2). The crystallin solution was decanted and filtered through a series of sterile Millipore membrane filters (5.0, 0.8, and 0.45 μm) (Millipore Corp., Bedford, MA). The solutions were stored under nitrogen at 4° until used. Protein concentrations were determined by the method of Lowry *et al.* (14).

Separation of Crystallins. Crystallin solutions were fractionated into α, β, and γ components by column chromatography using DEAE-cellulose (DE 52, Whatman, Kent, England) and a modification of the method of Spector *et al.* (15). The crystallin solution and the chromatography column were equilibrated at 4° with an initial buffer of 5 mM potassium phosphate (pH 6.8). The crystallin solution was applied to the column and was eluted with the initial buffer until the first peak, the γ-crystallins, were obtained. The β_1-, β_2-, and α-crystallin fractions were eluted by stepwise changes to 0.03 and 0.05 M potassium phosphate buffers (pH 6.8) followed by 0.5 M potassium phosphate buffer (pH 6.8) to which 0.25 M KCl had been added.

Incubation of Crystallin Solution. Sterile crystallin solutions (20–30 mg/ml) in 0.05 M potassium phosphate buffer (pH 7.3) were incubated in sealed tubes at 37°. [^{14}C]Glucose (5 mM, 50

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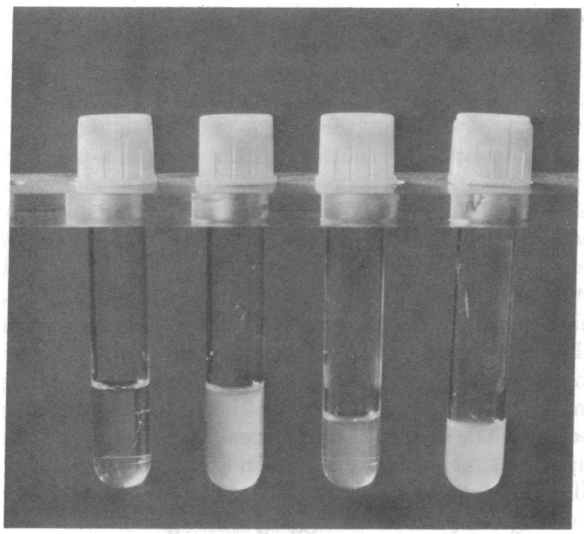

FIG. 1. Sterile solutions of bovine crystallin after 28 days of incubation at 37° with 5 mM glucose 6-phosphate or 50 mM glucose. From left to right: cortical, glucose; cortical, glucose 6-phosphate; nuclear, glucose; and nuclear, glucose 6-phosphate. At this time the control solutions (without hexose) showed no opalescence.

mM), [^{14}C]glucose 6-phosphate (5 mM; specific activity, 0.3, mCi/mol), or reducing agents (1 mM) in 0.05 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane buffer (pH 7.3) containing gentamicin (0.4 mg/100 ml) were added to test samples according to the experiment. The development of opalescence in these incubated solutions was monitored at 550 nm with a Coleman Jr. spectrophotometer.

The incorporation of radiolabeled substrate into acid-precipitable protein was monitored over time. Aliquots (50 μl) were removed periodically from tubes, diluted with 2 ml of distilled water, and precipitated with an equal volume of cold 10% trichloroacetic acid. The precipitated protein was collected on a Millipore membrane filter (5 μm) and washed with at least 15 ml of cold 5% trichloroacetic acid. The dried filters were placed in vials containing Aquasol (New England Nuclear) and the radioactivity was assayed in a Packard Tri-Carb liquid scintillation counter.

Gel Filtration Chromatography. The formation of high molecular weight aggregates after incubation of crystallin solutions with or without [^{14}C]glucose 6-phosphate was assessed by gel filtration chromatography. Samples (500 μl; 28 mg/ml) were applied at room temperature to a 50 × 1 cm Bio-Gel A-5m column that had been previously equilibrated with 0.05 M potassium phosphate buffer (pH 7.2) containing 0.02% sodium azide.

Borohydride Reduction. Crystallin solutions in 0.05 M potassium phosphate buffer (pH 7.0) were reduced with a 50–100 molar excess of sodium [^{3}H]borohydride (5 mCi/mol). The reaction proceeded for 10 min at room temperature and for an additional 50 min at 10° according to the method of Bookchin and Gallop (16). The solutions were extensively dialyzed at 5° to remove unreacted borohydride. The crystallins were then separated by ion exchange chromatography on DE 52 as described above. The amount of ^{3}H incorporated into acid-precipitable protein was determined as described above.

Adduct Analysis. The crystallins that were radiolabeled by [^{14}C]glucose incorporation or sodium [^{3}H]borohydride reduction were examined for the site of adduct formation. The α, β, and γ crystallins that had been separated by chromatography were hydrolyzed in 6 M HCl at 110° *in vacuo* for 8 hr. The HCl was evaporated under reduced pressure and the hydrolysate was chromatographed on an amino acid analyzer (Beckman no. 119C) (17). Chromatograms of the hydrolyzed labeled crystallins were compared with those of standard amino acids containing hydrolyzed N^6-1-(1-deoxyglucitolyl)-N^2-*t*-butoxycarbonyllysine. Division of the eluate stream permitted half of the fractions to be collected, dissolved in Aquasol, and assayed for radioactivity.

RESULTS

Initial experiments with sterile solutions of bovine crystallin revealed that the solutions remained clear *in vitro* for several months as long as they were stored under a nitrogen atmosphere with 5 mM dithioerythritol. In the absence of added reducing agents, the optical density (monitored at 550 nm) of the crys-

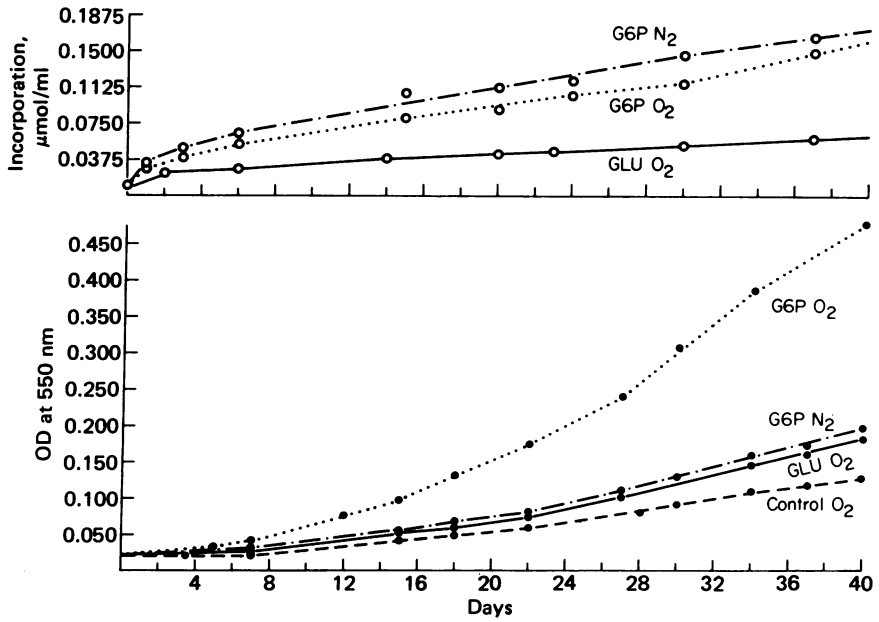

FIG. 2. Sterile solutions of bovine nuclear crystallins (50 mg/ml) in 0.05 M phosphate buffer (pH 7.3) were incubated alone or with ^{14}C-labeled glucose 6-phosphate (5 mM) or glucose (50 mM) under nitrogen (N_2) or air (O_2). (*Upper*) Incorporation of ^{14}C-labeled hexose into acid-precipitable protein as measured in 50-μl aliquots of the solution. (*Lower*) Optical density of crystallin solutions.

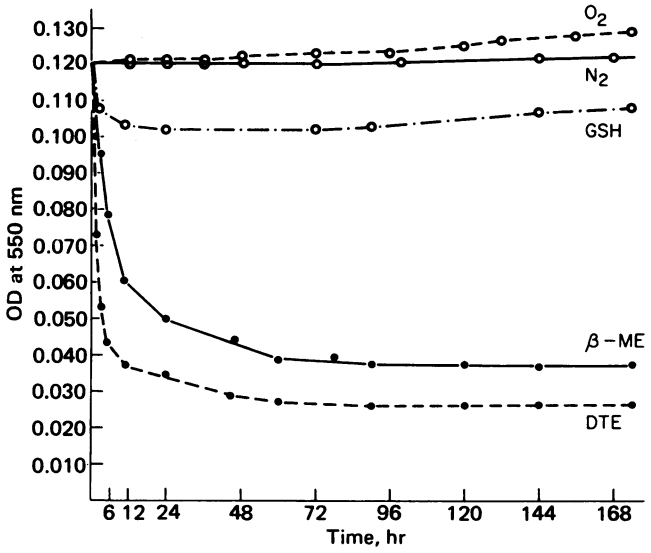

FIG. 3. Reversal of opalescence (induced by glucose 6-phosphate) in solutions of glycosylated bovine crystallins by reducing agents at 1 mM. Opalescent crystallins were mixed with buffered solutions of dithioerythritol (DTE), 2-mercaptoethanol (β-ME), or glutathione (GSH), gassed with nitrogen, and incubated at 37°. Control solutions of glycosylated crystallins were just gassed either with nitrogen (N_2) or air (O_2).

tallin solutions increased gradually over months even when the solutions were flushed with nitrogen; an aerobic environment hastened this process. This development of opalescence was greatly accelerated in crystallin solutions that contained 50 mM glucose or 5 mM glucose 6-phosphate in the presence of oxygen (Fig. 1).

Both [^{14}C]glucose and [^{14}C]glucose 6-phosphate were found, in time, to become associated with acid-precipitable proteins in the presence or absence of oxygen (Fig. 2 *upper*). The opacification of the crystallin solution, however, was not simply related to the amount of glycosylation. As shown in Fig. 2 *lower*, the solutions containing glucose 6-phosphate became opaque more rapidly in the presence of oxygen even though glycosylation had occurred to a comparable extent in the presence of nitrogen. It thus appears that the glycosylation of lens proteins with glucose or glucose 6-phosphate increased their susceptibility to sulfhydryl oxidation. This was supported by the observation that reducing agents not only could protect the solutions from becoming turbid but also could reverse much of the opalescence that had developed during the incubation (Fig. 3). In increasing order of effectiveness, glutathione, 2-mercaptoethanol, and dithioerythritol were able to reverse the opacity. In addition, sodium borohydride (not shown) was as effective as dithioerythritol in reversing the opacity.

The formation of high molecular weight aggregates in opalescent solutions of crystallins was investigated by gel filtration chromatography. An increase in aggregate formation occurred gradually in both the control and the glucose 6-phosphate samples after an initial lag period of 15 days. The elution patterns of the lens proteins after 1 and 41 days of incubation are shown in Fig. 4. After 41 days of incubation, both the control, which was incubated in absence of sugar, and the crystallin solution incubated with glucose 6-phosphate showed an increase in high molecular weight species (Fig. 4 *middle*, peaks I and II). However, compared with the control the sample incubated with glucose 6-phosphate had a larger increase in peak I (larger than 5×10^6 daltons) and peak II with a concomitant decrease in peak III. The material eluted in peak I (glucose 6-phosphate sample) was rechromatographed on a Sepharose 2B column. Half of the material loaded was eluted in the void volume, thus indicating the presence of aggregates larger than 4.0×10^7 daltons. The reversibility of the aggregation with reducing agents was examined by incubation of the solutions of lens proteins with 50 mM dithioerythritol for 60 min at room temperature prior to chromatography (Fig. 4 *bottom*). The reduction of the disulfide bonds induced a large decrease in peaks I and II and the regeneration of peak III. The reversibility of the control was more complete, thus indicating that the disulfide bridges of the glycosylated lens proteins may have not been completely reduced or denoting the presence of other intermolecular covalent linkages.

The site of glycosylation of crystallin proteins after incubation with [^{14}C]glucose or [^{14}C]glucose 6-phosphate was further investigated. In order to stabilize the bond formed between the sugar and protein, the solutions of crystallins were reduced with sodium borohydride. After extensive dialysis to remove unreacted borohydride, the crystallins were separated by chromatography. The amount of carbohydrate adduct for each of the crystallins is shown in Table 1. Under these conditions the carbohydrate was associated with all of the crystallins.

The relevance of these glycosylation reactions to intact lenses in culture and to diabetic animals was investigated. Isolated rat lenses that were cultured *in vitro* in a high glucose concentration (30 mM) developed, in time, an opacity that mimicked cataracts. The crystallins isolated both from these cultured lenses and from control lenses were reduced with sodium [^{3}H]borohydride and chromatographed. The α-crystallins of the lenses maintained in the high-glucose environment had a greater amount of radioactivity, indicating an increased number of borohydride-reducible bonds (Table 2). The increase in borohydride-reducible bonds per mg of protein per day of

Table 1. Incorporation of ^{14}C-labeled hexoses into crystallins after 14-day incubation

	5 mM glucose 6-phosphate		50 mM glucose	
Crystallin	cpm/mg	nmol/mg protein	cpm/mg	nmol/mg protein
		Cortical crystallins		
γ	375.2	1.0	254.6	0.6
β_1	470.6	1.25	811.6	1.8
β_2	437.0	1.16	343.9	0.8
α	483.7	1.29	730.8	1.7
		Nuclear crystallins		
γ	856.0	2.28	289.4	0.90
β_1	842.0	2.24	372.8	0.87
β_2	708.0	1.89	365.1	0.85
α	825.9	2.20	290.1	0.675

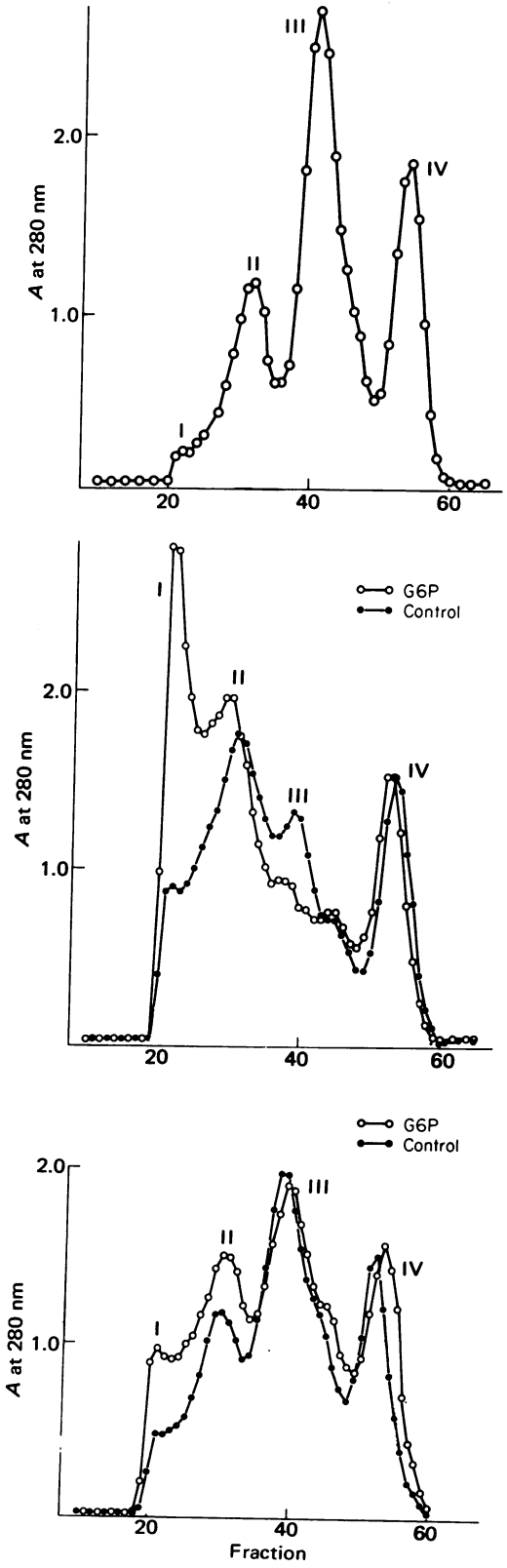

FIG. 4. Chromatography (on Bio-Gel A-5m) of bovine nuclear crystallins incubated at 37° alone or with 5 mM glucose 6-phosphate (G6P). The elution rate was 4.1 ml/hr; 0.6-ml fractions were collected. The void volume was determined with blue dextran which eluted in fraction 20. (*Top*) Day 1. (*Middle*) After 41 days of incubation. (*Bottom*) After 43 days of incubation followed by reduction with 50 mM dithioerythritol. The column was equilibrated with 50 mM dithioerythritol.

Table 2. ^3H incorporation in reduced crystallins from cultured rat lenses

Crystallin	^3H, cpm/mg protein*	
	Control	6-Day culture
γ	3,717	3,208
β	13,488	12,134
α	13,136	18,976

* A variability of up to 1000 cpm may be attributed to exchangeable or residual ^3H.

culture was approximately the same (900 cpm/mg per day) for 7 and 11 days of culture duration, indicating a linear increase in glycosylation.

A similar increase in borohydride-reducible bonds was found to be associated with the crystallins of cataractous lenses isolated from diabetic rats. Table 3 shows the incorporation of ^3H into the various crystallins isolated from normal and cataractous lenses. It appears that the crystallins from the cataractous lens have more adducts. The decrease in the specific activity of the γ-crystallin fraction may reflect a selective precipitation of γ-crystallins. Evidence that the incorporated ^3H was in fact associated with a glyco moiety of the α-crystallins was obtained by detection of glycosylated lysine residues. The α-crystallins isolated from cataractous lenses that had been reduced with sodium [^3H]borohydride were acid hydrolyzed and subjected to amino acid analysis. These α-crystallins contained a novel ninhydrin-positive material (peak A) that contained ^3H and eluted from the column at the same position as acid-hydrolyzed N^6-1-(deoxyglucitolyl)lysine (Fig. 5). It was estimated by planimetry that approximately 10% of the lysines were glycosylated in this sample. ^3H-Labeled ϵ-glycosyllysine has also been found in the acid hydrolysate of β and γ crystallins of the diabetic rat. However, it has not been detected in comparable amounts of hydrolysates of crystallins isolated from normal rat lenses.

DISCUSSION

The glycosylation of ϵ-amino groups of lysine in the crystallin proteins of the diabetic rat lens offers a new mechanism for the formation of cataracts. This modification of crystallins by glucose or glucose 6-phosphate was shown to confer on the proteins an increased susceptibility to disulfide bond formation which resulted in an increased absorbance of light and an increased formation of high molecular weight aggregates. Previous studies on cataractous lenses have implicated both sulfhydryl oxidation and high molecular weight aggregates as involved in cataractogenesis (18–24). For example, the maturation of cataracts has been shown to be correlated with a decrease in free sulfhydryl groups and an increase in the amount of urea-insoluble proteins; both changes were attributed to disulfide bond formation (22–24).

The increased susceptibility of crystallin sulfhydryl groups to oxidation after the glycosylation of lysine residues may be explained in part by the observations of Spector and Zorn (25). They found an increase in titratable sulfhydryl groups of α-

Table 3. ^3H incorporation in reduced crystallins from rat lenses

Crystallin	^3H, cpm/mg protein	
	Normal	Diabetic
Unseparated homogenate	3215	15,259
γ	3512	1,941
β_1	2967	3,551
β_2	4387	6,260
α	2646	19,155

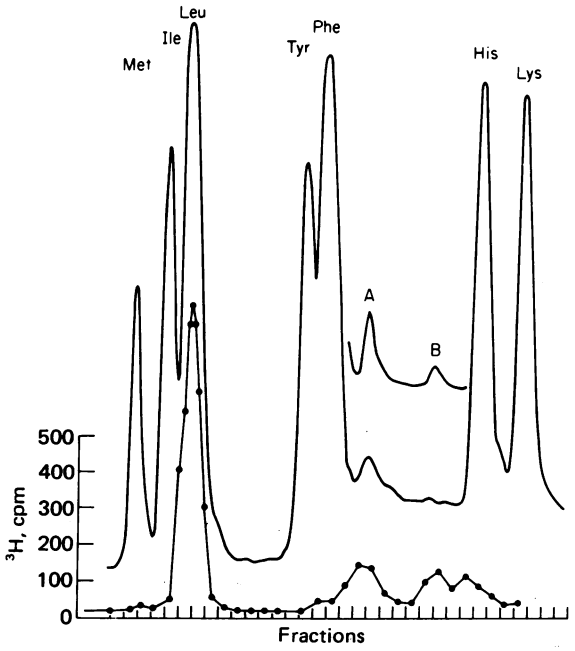

FIG. 5. A portion of the chromatogram of acid-hydrolyzed rat α-crystallin isolated from a diabetic, cataractous lens and reduced with NaB[^{3}H]H$_4$. Half of the eluate was utilized for the chromatogram of ninhydrin-positive material and half for the profile of ^{3}H counts (●) graphed below it. A small amount of [^{3}H]leucine was added to the hydrolysate in order to align the chromatogram with the profile of radioactivity. A significant increase in ^{3}H was found to coincide with two small peaks of the chromatogram. Above the chromatogram is the tracing of the eluate of hydrolyzed, chemically synthesized N^6-1-(1-deoxyglucitolyl)lysine. A, elution position of the native compound alone; B, a conversion product that accumulates progressively during acid hydrolysis.

crystallin as the pH was increased from 8.8 to 10.4. It was proposed that the deionization of the ε-amino groups of the lysine residues altered the conformation of the protein, resulting in an unmasking of the sulfhydryl groups.

The accumulation of sorbitol in the lens has also been proposed as a mechanism for sugar-induced cataract formation (26). A high-glucose environment is associated with an increased formation of sorbitol through the reduction of glucose by aldose reductase (26, 27). The inhibition of this enzyme has been the basis for developing drugs to prevent cataracts (28). However, we would interpret the results obtained with aldose reductase inhibitors as occurring either by their activity as free-radical trapping agents or through a sparing effect on the NADPH pool. The concentration of NADPH may be crucial in maintaining the reduced sulfhydryl groups because it is a cofactor for glutathione reductase.

The nonenzymatic glycosylation of lens proteins described above and the glycosylation of hemoglobin A (8, 9) are early stes in the Maillard reaction or "nonenzymatic browning" of proteins that occurs with aging (29, 30). In this reaction, various reducing sugars (e.g., glucose, galactose, fructose, ribose) form adducts with the amino groups of amino acids, peptides, and proteins. It is believed that, subsequent to Schiff base formation, the aldamine or ketoamine undergoes an Amadori or Heyns rearrangement, respectively, to form stable products. The carbonyl groups of these rearrangement products can in turn undergo further addition with other amino groups to form disubstituted sugars and possibly multisubstituted sugars (31). Alternately, the glyco group can undergo an internal rearrangement and release furfuraldehyde which can also react with amino groups (32). In the later stages, depending on the conditions (e.g., temperature, pH, type of amino acids and sugars), compounds are formed that are fluorescent and yellow to brown (33). This suggests that the fluorescent yellow and brown colors seen in human cataracts (34) could be derived from the later steps of the nonenzymatic browning of glycosylated crystallins.

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