Silver staining of collagen type I after sodium dodecylsulphate polyacrylamide gel electrophoresis: effect of Maillard reaction

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ABSTRACT

Differences in the acidic silver staining, after sodium dodecylsulphate polyacrylamide gel electrophoresis, between the $\alpha 1$ and $\alpha 2$ collagen chains, as well as between rat-tail tendon and calf-skin collagen type I, were observed. The staining conditions at which the staining differences are both most expressed and reproducible were characterized. Age differences between staining of the $\alpha 1$ CB6 fragment from young rats (2 and 12 months) and old rats (29 months) indicated that different susceptibilities of collagen species to the silver staining can be the result of different extents of some age-dependent post-translational modification, such as glycation. *In vitro* incubation of acid-soluble rat-tail tendon collagen with various sugars led to an increase in silver staining compared with samples incubated in the absence of sugar. This effect was inhibited by sodium cyanoborohydride, diethylenetriamine pentaacetic acid and aminoguanidine, *i.e.* compounds inhibiting the Maillard reaction at various stages. It can be concluded that the enhanced silver susceptibility of glycated collagen is related to advanced-phase Maillard reaction products attached to collagen.

INTRODUCTION

Silver staining is a widespread, high-sensitivity detection technique used after electrophoretic separation of macromolecules. Although the mechanism is not yet fully understood, it is generally agreed that some amino acid side-chains of proteins take part in silver binding (for a review see ref. 1) and that subsequent reduction of these protein–silver complexes leads to the formation of metallic silver deposits in protein zones, which are seen as bands or spots in a gel. Differences in protein amino acid composition are suggested to be responsible for the specific staining behaviour of individual proteins [2]. Variations in silver staining conditions may enhance the ability to bind silver by certain groups and suppress the binding by others (*e.g.* a dominant role of carboxyl groups in acidic silver staining methods and the role of amino groups in alkaline methods [1]).

Moreover, it is probable that changes of silver binding groups by covalent modification can affect the silver staining of a particular protein. Sammons *et al.* [3] proposed that post-translational modifications of proteins may be the cause

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of the production of various colours observed under certain staining conditions. Dzandu et al. [4] reported a silver staining technique offering specific coloured detection of glycoproteins and sialoglycoproteins. It was suggested [5] that O-glycosidically linked carbohydrate moieties are responsible for the characteristic yellow colour and N-linked moieties for the brown colour of protein bands of some glycoproteins. Another "specific" silver staining was previously reported for proteins containing dihydroxyphenylalanine [6]. Thus modified silver staining can allow more or less specific detection of various groups localized on a protein molecule. Moreover, it can be assumed that other chemical groups not derived from amino acid side-chains, but attached to the protein after its translation, may have the ability to interfere with the staining mechanism.

This study was initiated by the observation that silver staining of collagen α1 and α2 chains after sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) is strongly dependent on the staining method used. The aim was to find the cause of differences in the silver staining of collagen α chains. The amino acid compositions of collagen α1 and α2 chains differ only minimally, and it was therefore of interest to explain their different stainabilities. The staining susceptibilities of the two chains may reflect differences in the degree of enzymic (e.g. glycosylation, hydroxylation), non-enzymic (e.g. glycation, free radical-mediated amino acid oxidation) and other post-translational modifications of collagen α chains. It was demonstrated that this effect may be related to changes in protein structure caused by glycoxidation. We characterized staining conditions under which the differences are clearly expressed and reproducible, and an attempt is made to explain the chemical background of this phenomenon.

EXPERIMENTAL

Chemicals

SDS, glycine, N,N,N′,N′-tetramethylethylenediamine, phenylmethylsulphonyl fluoride (PMSF), Coomassie Brilliant Blue R-250 (CBB),

urea and sodium cyanoborohydride (NaCNBH$_3$) were purchased from Serva (Heidelberg, Germany); diethylenetriamine pentaacetic acid (DETAPAC), and DL-glyceraldehyde were obtained from Sigma (St. Louis, MO, USA); acrylamide and N,N′-methylenebisacrylamide were from Bio-Rad (Richmond, CA, USA); aminoguanidine bicarbonate was a product of Koch-Light (Colnbrook, UK); N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) was from Calbiochem (Los Angeles, CA, USA); sodium borohydride (NaBH$_4$) was purchased from Metallgesellschaft (Frankfurt, Germany). All other chemicals were of analytical-reagent grade.

Sample preparation

Acid-soluble calf-skin collagen type 1 was extracted and purified as previously described. After removal of the neutral salt-soluble fraction [7], collagen type III was removed by salt precipitation with 1.7 M NaCl. Acid-soluble collagen (ASC) from the tail tendon of rats 2, 3, 12, and 29 months old was prepared according to the procedure of Chandrakasan et al. [8].

Glycation

Lyophilized acid-soluble tail tendon collagen from rats 3 months old was dissolved in 10 mM acetic acid (3 mg/ml). Then the collagen sample was mixed 1:1 with sodium phosphate buffer, pH 7.5 (buffer A, 100 mM final concentration) or with HEPES buffer, pH 7.5 (buffer B, 25 mM HEPES, 25 mM KCl, 0.5 mM PMSF final concentration) without sugars (control) or with various concentrations of glucose, fructose, ribose and glyceraldehyde as specified in the legends to each figure. Some samples were glycated by ribose or glyceraldehyde in the presence of 1 mM DETAPAC, 125 mM aminoguanidine (in the case of ribose) or 25 mM aminoguanidine (in the case of glyceraldehyde), and with 125 mM NaCNBH$_3$, respectively. Incubations were carried out for 24 h at 21°C in the case of glyceraldehyde or for various time periods at 37°C in the case of other sugars, as specified in the figure legends. Microbial growth was prevented by the addition of toluene (1 μl/ml) to each incubation

mixture. Some samples were reduced after incubation with a 200 molar excess of $NaBH_4$ for 1 h at room temperature.

Cyanogen bromide (CNBr) cleavage

About 10 mg of lyophilized ASC were dissolved in 1 ml of 70% formic acid. A 100-mg quantity of CNBr was added, and the mixture was kept at room temperature for 24 h. The resulting peptides were vacuum-dried by five times repeated dissolution in distilled water and subsequent lyophilization.

SDS-PAGE

SDS-PAGE was carried out according to Laemmli [9] on 6% or 7.5% gels for collagen chains or on 12% gels for CNBr peptides. Gel thickness was 1 mm, or as specified in the figure legends. Samples for analysis were prepared as follows: (1) by dissolving lyophilized proteins in sample buffer [2.3% (w/v) SDS, 10% (v/v) glycerol, 125 mM Tris–HCl, pH 6.8], and heating them to 95°C for 5 min; (2) by mixing aliquots of the incubation mixture 1:1 with 4.6% (w/v) SDS, 20% (v/v) glycerol, 250 mM Tris–HCl solution (pH 6.8), and heating them to 60°C for 30 min or to 95°C for 5 min. Some samples were mixed with 8 M urea (1:1) and loaded on the gel without heating.

Gel staining

Silver staining was performed according to Blum et al. [10], Merril et al. [11], Sammons et al. [3] and Morrissey [12] without modifications. A glycation-sensitive procedure was adapted from Morrissey's method [12] by omitting the DTT step (for the steps of the adapted procedure see Table I). The effect of various enhancing agents was tested by replacing the glutaraldehyde solution (step III in Table I) with one of the following reagents: 3.4 mM $K_2Cr_2O_7$–3.2 mM HNO_3 solution (10 min incubation), 2% periodic acid solution (10 min), 0.1% $NaBH_4$ (30 min), or 0.4% sodium thiosulphate (1 min), with appropriate adjustment of subsequent washing before the silver impregnation step.

Control gels were fixed and stained with 0.05% CBB in 40% methanol–8% acetic acid–water for 2 h or overnight, and destained in 25% methanol–8% acetic acid–water.

Densitometric measurements were performed on a Shimadzu CS-930s thin-layer chromatography scanner (Shimadzu, Tokyo, Japan) at 560 nm for CBB-stained gels and at 450 nm for silver-stained gels.

TABLE I

MODIFIED SILVER STAINING PROCEDURE, ADOPTED FROM MORRISSEY'S PROTOCOL

Gels were 1 mm thick.

I	Fixation	100 ml of 50% MeOH–10% acetic acid	30 min
II	Washing	100 ml of 5% MeOH–7% acetic acid	30 min
III	Enhancement	50 ml of 10% glutaraldehyde	30 min
IV	Washing	500 ml of distilled water	3 × 10 min
			3 × 20 min
V	Silver impregnation	100 ml of 0.1% silver nitrate	20 min
VI	Rinse	100 ml of distilled water	1 min
VII	Development	50 ml of 0.185% formaldehyde–3% sodium carbonate	3 × 20 s
		100 ml of 0.185% formaldehyde–3% sodium carbonate	5–10 min
VIII	Stop	Add 2.3 M citric acid to neutral pH	10 min
IX	Washing	500 ml of distilled water	3 × 10 min

RESULTS

Silver staining of collagen α chains by conventional procedures

Differences in the silver stainability of acid-soluble rat-tail tendon type I collagen α1 and α2 chains strongly depend on the staining method used. Fig. 1 shows densitograms of collagen type I separated by SDS-PAGE and stained either by the silver staining procedures of Merril et al. [11]
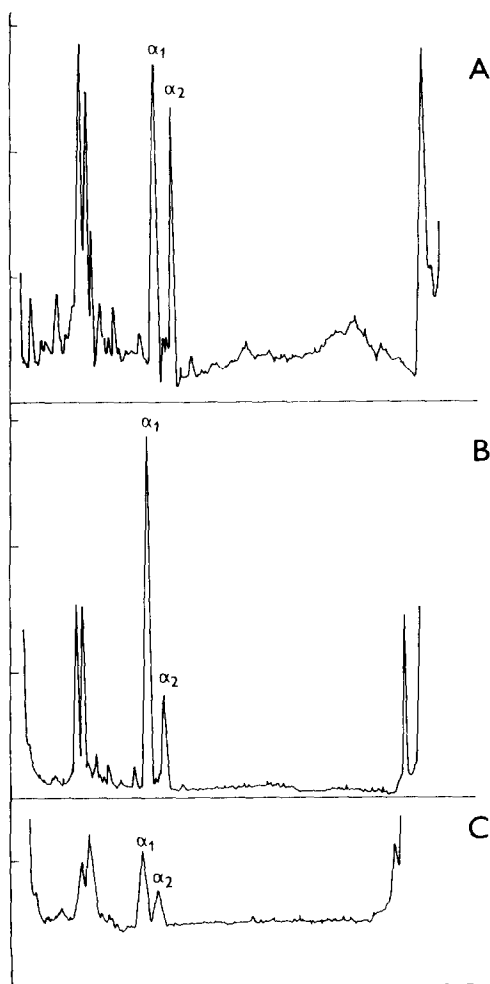

Fig. 1. One-dimensional densitograms of gels (7.5% T, 1.5 mm thick) stained with silver according the methods of Merril et al. [11] (A) and Blum et al. [10] (B), and with CBB (C); α chains of ASC are indicated as α1 and α2 on the top of the corresponding peak. The α1/α2 area ratio is 1.6:1 for (A), which has 0.25 μg of collagen per lane, 4.3:1 for (B), which has 1.25 μg of collagen per lane, and 2:1 for (C), which has 12.5 μg of collagen per lane.

and Blum et al. [10] or with CBB. It is known that the ratio of the staining intensities of α1/α2 collagen chains after CBB staining is 2:1, in agreement with the current knowledge that collagen type I consists of two α1 and one α2 polypeptide chains.

In our determinations, the ratio of the staining intensities of α1 and α2 collagen chains was ca. 1.8:1 for calf-skin collagen and ca. 2.2:1 for rat-tail tendon collagen. However in silver staining the α1/α2 ratio varied markedly with the method used. Generally, in the methods sensitive for collagen polypeptides, e.g. the procedure of Sammons et al. [3], (not shown) and Merril et al. [11] (Fig. 1A), the α1/α2 ratio approaches 1.5:1 for a broad range of protein loading (0.25–25 μg per lane) with a tendency to shift to 1:1 with increased loading. Such a difference in α chain staining cannot be explained simply by faster saturation of the α chain zone with silver ions at low levels of loading as then a saturation effect is unlikely to occur. On the other hand, the saturation effect is responsible for shifting the ratio from 1.5:1 to 1:1 with increased collagen sample loading per lane.

The opposite situation occurs when deploying methods relatively insensitive to collagen, e.g. the procedure of Morrissey [12] (not shown) or Blum et al. [10] (Fig. 1B). The α1/α2 ratio for these methods is much higher than in the case of CBB staining. For Blum's method the α1/α2 ratio was 4:1 at 0.2 μg of collagen per lane and 6:1 at 2.5 μg of collagen per lane. Sodium thiosulphate, which was used in this method as an enhancer, had a small effect on the collagen staining sensitivity (see also Table II). For a number of other proteins the sensitivity of this method was substantially higher and the sensitivity range reported in the literature was easy to reproduce. When applying the method of Morrissey [12] the α1/α2 ratio was ca. 3:1 and was strongly dependent on he dithiothreitol (DTT) concentration in the enhancing solution. We also examined the extent to which commonly used silver staining enhancers influence collagen staining. Data presented in Table II show the staining sensitivities of different methods, as well as the differences in staining intensity between α1 and α2 collagen chains. Of all

TABLE II

EFFECT OF SOME SILVER STAINING ENHANCERS ON COLLAGEN STAINING SENSITIVITY AND ON EXPRESSION OF DIFFERENCES IN CHAIN STAINING

Enhancer	Sensitivity to collagen	Diferences in $\alpha1/\alpha2$ chains staining
Glutaraldehyde	Moderate	+ +
DTT–glutaraldehyde[a]	High	+
$K_2Cr_2O_7$–HNO_3	Moderate	+ +
Periodic acid	High	Not observed
Sodium thiosulphate	Moderate	+ + +
Sodium borohydride	High	Not observed
Sammons et al. method[b]	High	Not observed

[a] Morrissey's original procedure [12].
[b] No special enhancing step is used in this method [3].

the modifications tested, only the procedure adapted from Morrissey's approach [12] (Table I) resulted in relatively monochromatic staining; the method of Blum et al. [10] resulted in a variety of colours depending on sample loading. In all following experiments the modified method of Morrissey [12] was used: namely omitting DTT from the enhancing solution. When the enhancing step (i.e. treatment by glutaraldehyde solution, step III in Table I) was omitted from the adapted Morrissey's procedure, discriminate staining of $\alpha1$ and $\alpha2$ collagen zones resulted as well, although at a considerably lower level of sensitivity. Thus glutaraldehyde appeared unnecessary for the expression of differentiated staining of collagen $\alpha1$ and $\alpha2$ chains.

Differences in CBB and silver staining of collagen type I of different origins

It was demonstrated that the ratio of staining intensity of $\alpha1$ and $\alpha2$ chains is not the same for the rat-tail tendon and calf-skin collagen type I. (See Fig. 2A for CBB staining and Fig. 2B for modified silver staining.) Densitometric measurements revealed the ratio 2.2:1 for rat-tail tendon and 1.8:1 for calf-skin collagen type I in CBB staining, whereas modified silver staining revealed a ratio of 4.3:1 for rat-tail tendon and 33:1 for calf-skin type I collagen. CNBr-derived fragments of acid-soluble collagen from rat-tail tendon of rats 2, 12, and 29 months old were ana-

lysed to identify further the part of the collagen molecule particularly sensitive to silver staining changes. It was demonstrated that this fragment is the $\alpha1$ CB6 peptide. In the 29-month-old rat this peptide is stained much more intensively
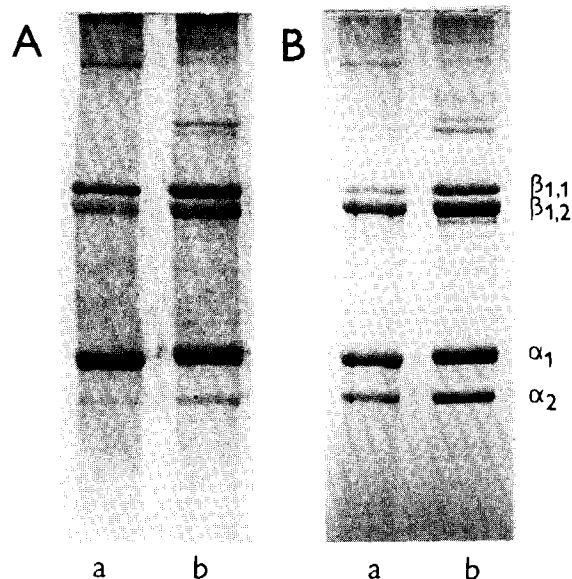

Fig. 2. Differences in modified silver staining (A) and CBB staining (B) between collagen type I of different origins. Lane a is acid-soluble calf-skin collagen (20 μg per lane), and lane b is acid-soluble rat-tail tendon collagen (30 μg per lane). The positions of collagen monomers and dimers (α_1 and α_2; $\beta_{1,2}$ and $\beta_{1,1}$) are indicated. Densitometric measurements of both stained gels revealed the $\alpha1/\alpha2$ ratio was 1.8:1 for calf collagen and 2.2:1 for rat collagen on CBB staining, and 33:1 for calf collagen and 4.3:1 for rat collagen on silver staining.

than the corresponding fragment from rats 2 or 12 months old (Fig. 3).

Modified silver staining of collagen after in vitro glycation

The non-helical domain of the α1 CB6 fragment is the site where naturally occurring [13] as well as sugar-derived cross-links [14] are formed; it was assumed that the enhanced staining of this site of the collagen molecule may be mediated by a product related to the Maillard reaction. To verify this, the acid-soluble rat-tail tendon collagen of 3-month-old rats was incubated with various sugars, as glucose, fructose, ribose and glyceraldehyde. This treatment leads to an increase in the staining of both α chain bands (mainly of α1) as well as α chain polymers (β and γ bands) of collagen (*e.g.* Fig. 4, lane d). The increase in the staining intensity was dependent on the nature of the sugar used, its concentration and time of in-

Fig. 4. Modified silver staining (A) and control CBB staining (B) of ASC glycated *in vitro*. The gels were loaded with acid-soluble collagen (25 μg), which was incubated for 3 days at 37°C in buffer B alone (lane a), in the presence of 500 mM D-glucose (lane b), 500 mM D-fructose (lane c) and 500 mM D-ribose (lane d), respectively.

cubation; it correlated well with the known reactivity of the particular sugar (Fig. 4). Intensity changes in the staining pattern of collagen CNBr-derived fragments (Fig. 5) are analogous to the radioactive labelling affinity results of CNBr fragments on autoradiograms obtained by incubating rat-tail tendons with radioactive ribose [15]. The α1 CB3, α1 CB8 and α2 CB3-5 fragments were stained after *in vitro* glycation relatively less than the α1 CB6, α1 CB7, α2 CB4, α1 CB6-7 and α1 CB3-7 fragments.

Silver staining of collagen glycated in the presence of sodium cyanoborohydride, aminoguanidine and diethylenetriamine pentaacetic acid

If the susceptibility of collagen to silver staining is indeed mediated by glycation or glycoxidation, it should be blocked by agents that inhibit the Maillard reaction. NaCNBH₃ selectively reduces the double bond of a Schiff base, and thus prevents the formation of the Amadori product through double bond rearrangement. If present in our experiments, it blocked practically all the staining changes in collagen incubated with ribose (Fig. 6, lane e). Staining changes were also partially inhibited by the use of glyceraldehyde, probably because of its much higher reactivity (Fig. 7, lane e). Incubation of a sugar–collagen
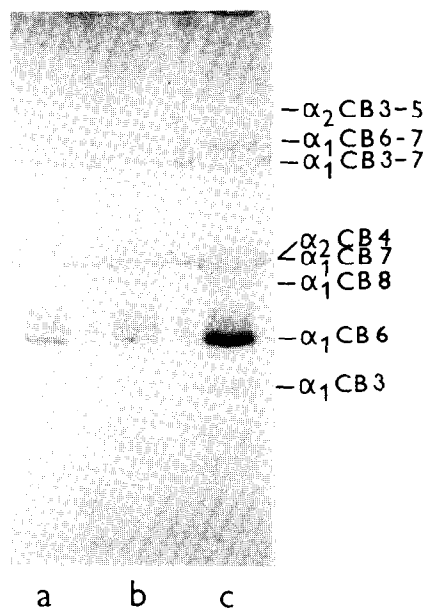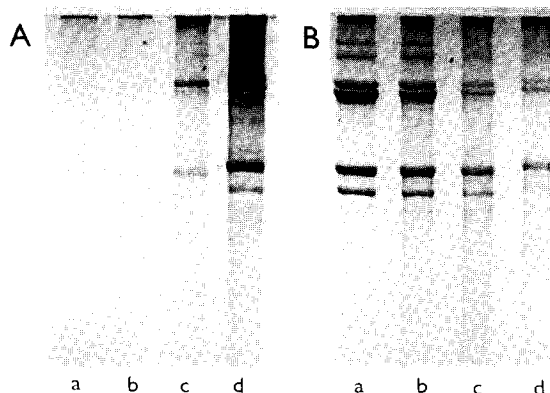

Fig. 3. Modified silver staining of CNBr-derived collagen fragments from rats of different ages. The gel containing CNBr fragments derived from ASC from rats 2 (lane a), 12 (lane b), and 29 months (lane c) old. The positions of the individual fragments are indicated, and correspond to the positions of fragments in the second part of the same gel stained with CBB. A remarkable increase in staining intensity is seen for the α1 CB6 fragment from 29-month-old rats. One typical gel from three independent experiments is shown.
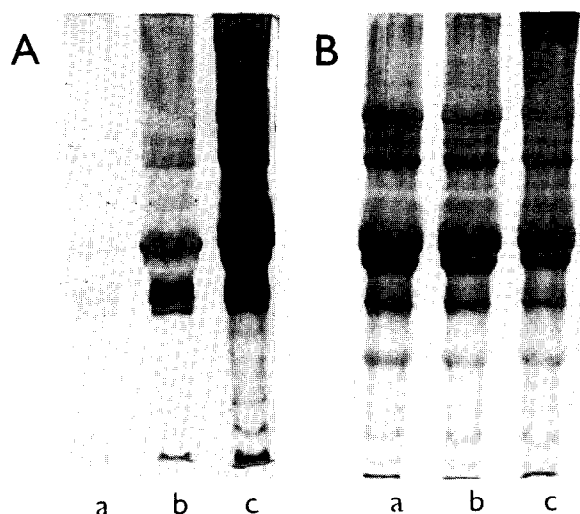
Fig. 5. Modified silver staining (A) and control CBB staining (B) of CNBr-derived fragments of collagen incubated with glucose for various times. Each lane contained the CNBr fragments (25 µg per lane) of acid-soluble rat-tail tendon collagen incubated at 37°C either in buffer A alone (control) for 6 days (lane a), or in the presence of 100 mM D-glucose for 3 days (lane b) and for 6 days (lane c).

mixture with aminoguanidine (which probably reacts with 1,2-dicarbonyls and Amadori products formed during the Maillard reaction) also inhibited positive staining (Fig. 8, lane c for ribose), and partially inhibited staining in the case of glyceraldehyde (Fig. 7, lane g). The reduction of collagen by $NaBH_4$ after incubation with sugar had no substantial effect on the staining of glycated or non-glycated collagen (Fig. 7, lane d for glyceraldehyde; Fig. 6, lane d for ribose: only positive results are presented).

The metal-chelating agent DETAPAC inhibited positive staining if collagen was incubated with sugar in its presence (Fig. 6, lane c for ribose; Fig. 7, lane c for glyceraldehyde). Control addition of DETAPAC to a glycated and a non-glycated sample before the electrophoretic run had no effect on the final staining of either sample (Fig. 7, lane h: only the glycated sample is shown).

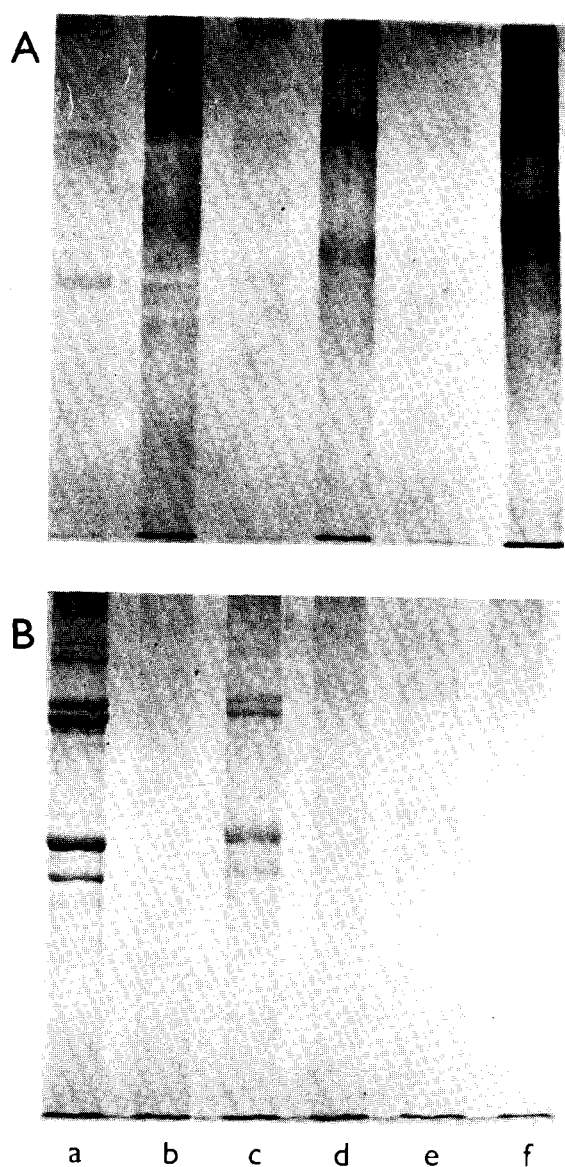The unknown product participating in enhanced staining is not formed during heating of

Fig. 6. Effect of collagen glycation with ribose in the presence of DETAPAC, $NaCNBH_3$ on silver staining (A) and on control CBB staining (B). ASC was incubated for 40 h at 37°C in buffer A (control, lane a), or with 125 mM D-ribose (lane b), with 125 mM D-ribose in the presence of 1 mM DETAPAC (lane c), and after reduction with $NaBH_4$ (lane d), with 125 mM D-ribose in the presence of 125 mM $NaCNBH_3$ (lane e). With the exception of the control sample (a), none of these samples was solubilized completely after being dissolved and heated in the electrophoretic sample buffer (owing to extensive cross-linking by long-term glycation); 25 µl of each incubation mixture (i.e. 25 µg of collagen of control sample) were loaded per lane.
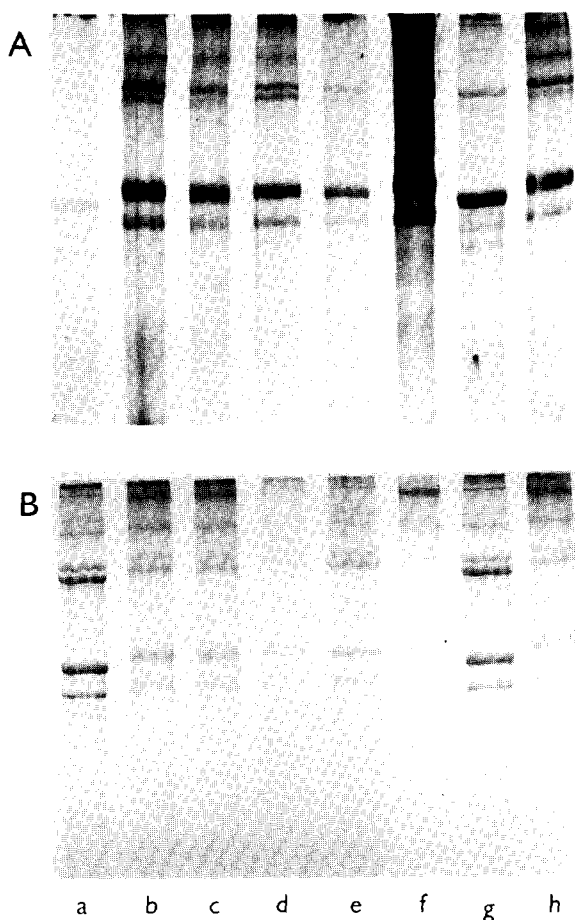
Fig. 7. Effect of collagen glycation with glyceraldehyde, in the presence of DETAPAC, AG and NaCNBH$_3$, on silver staining (A) and on control CBB staining (B). Both gels contained ASC (25 μg per lane), which was incubated for 24 h at 21°C as follows: in buffer A alone (lane a), or with 25 mM of DL-glyceraldehyde (lane b), with 25 mM DL-glyceraldehyde in the presence of 1 mM DETAPAC (lane c), with 25 mM DL-glyceraldehyde and after NaBH$_4$ reduction (lane d), with 25 mM DL-glyceraldehyde in the presence of 125 mM NaCNBH$_3$ (lane e), with 25 mM DL-glyceraldehyde solubilized in 4 M urea (final concentration, lane f), with 25 mM DL-glyceraldehyde in the presence of 25 mM aminoguanidine (lane g), with 25 mM DL-glyceraldehyde after addition of DETAPAC (1 mM final concentration before the sample loading) (lane h). Note that CBB stainability was influenced by *in vitro* glycation; this phenomenon was not further investigated.

protein in electrophoretic sample buffer. Surprisingly, the staining intensity was substantially increased, by an unknown mechanism, when collagen samples were heated to 60°C only (Fig. 6, lane f) or denatured by 4 M urea without any heating (Fig. 7, lane f).
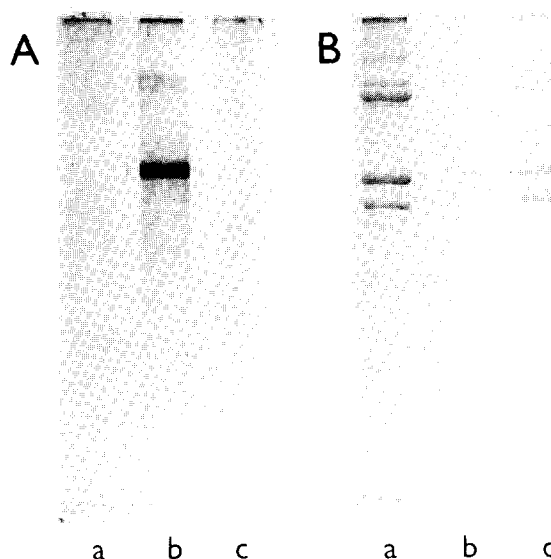

Fig. 8. Effect of collagen glycation by ribose in the presence of aminoguanidine on silver staining (A) and on control CBB staining (B). ASC (25 μg per lane) was incubated in buffer A alone (lane a), with 125 mM D-ribose (lane b) or with 125 mM ribose and 125 mM aminoguanidine (lane c) for 24 h at 37°C.

DISCUSSION

Detailed studies on the mechanism of the silver staining reported previously [16–18] suggested that the behaviour of a particular protein is determined by the type and content of the silver-reactive groups (*i.e.* amino acid residues) present in a protein. Their spatial arrangement and the cooperative effect expressed in silver ion reduction are also suggested to play a role in the staining mechanism [18].

The silver stainability differences for both collagen α chains observed by us may belong in this category, *i.e.* they may originate from the different amino acid compositions of α1 and α2 chains and their different conformations in the gel. For the amino acid compositions (bovine skin collagen type I) of the α1 and α2 chains presumably taking part in silver staining mechanism, the following ratios (α1/α2 per 1000 residues) have been reported [19]: 45:47 residues for aspartate; 77:71 residues for glutamate; 7:4 residues for methionine; 32:21 residues for lysine; 5:11 residues for hydroxylysine; 3:8 residues for histidine; and

50:57 residues for arginine. These differences do not appear significant and, in our opinion, are unlikely to play a crucial role in the differential staining of the two collagen chains under the conditions of acidic staining. Moreover, it is difficult to explain, on this basis, the inter-species differences in collagen type I silver stainability (see the results from calf and rat collagen) as the differences in amino acid composition are much smaller between bovine and rat $\alpha 1$ chains than between $\alpha 1$ and $\alpha 2$ chains from the same species [20]. There are, however, chain differences and inter-species differences in the extent of post-translational processing, *i.e.* in proline and lysine hydroxylation as well as hydroxylysylglycosylation [21,22]. Furthermore, the fact that CNBr fragments of collagen from 2-month-old rats are stained almost uniformly, but obvious differences are obtained when staining the $\alpha 1$ CB6 fragments from 2- and 29-month-old rats, indicates that differential staining effects will not depend on differences in amino acid composition only. This suggests that these age-dependent differences in the staining of the $\alpha 1$ CB6 fragment may be due to some post-translational modification, such as glycation, some products of which are known to accumulate in long-lived proteins with advancing age of the organism (*e.g.* pentosidine [23,24], carboxymethyllysine and carboxymethylhydroxylysine [25,26], the only ones yet structurally identified).

The experiments on *in vitro* incubation of collagen with various sugars support this idea. The mechanism of glycation-mediated silver staining enhancement can be based on the increased number of silver-binding or silver-reducing groups on protein.

The chemistry of the Maillard reaction is highly complex [27] and not yet resolved in all details. A number of new chemical groupings originating from this reaction are likely candidates for interfering with and altering the staining mechanism. For example, carbonyl and carboxyl groups (generally arising at various stages of the Maillard reaction, including Amadori rearrangement products) are assumed to participate under acidic silver staining conditions [1]. These groups

can be formed in two parallel ways: they can arise from the chemical modification of a protein–sugar adduct [28] or they can occur as a result of a free radical modification of some amino acids (*e.g.* proline [29] or histidine [30,31]; for a review see ref. 32). The latter possibility is less likely because it was observed that CNBr-derived fragments of collagen incubated in the presence of ascorbate or in the presence of H_2O_2/Cu^{2+} showed no silver staining differences (unpublished results).

The binding capacity of the enediol form of the Amadori product for heavy metals, *e.g.* for Cu^{2+} [33], Fe^{3+} [34] or iodine, offers another possible explanation for the increased sensitivity of glycated proteins to silver staining. Literature data concerning the role of aminoguanidine [35], DETAPAC [36–38] or $NaBH_4$ [39,40] indicate that ketoamine is not the main participant in the silver staining of glycated proteins. (Neither can aldimine be responsible for the described staining effect, because extensive dialysis of the sample before electrophoresis had no effect on the final silver staining of glycated collagen.)

Based on the results obtained with the inhibitory effect of DETAPAC, aminoguanidine, and $NaCNBH_3$, it may be proposed that increased susceptibility of glycated collagen to silver staining depends either on the generation of oxygen free radicals (catalysed by transition metals) or (with great probability) on the production of dicarbonyl compounds formed during the Maillard reaction.

Whatever the reason may be, the changes in silver stainability of the *in vitro* glycated protein reflect a protein modification related to the involvement of advanced-stage Maillard reaction products. It is interesting that we have not found substantial differences in the level of glycation for collagens obtained from animals of various ages measured by tetrazolium nitroblue or thiobarbituric acid assays. This indicates either that neither of these two assays is sensitive enough to reveal subtle differences in glycation or that silver staining and these assays involve different compounds.

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