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A Transport Form of Collagen from Embryonic Tendon: Electron Microscopic Demonstration of an NH_2-Terminal Extension and Evidence Suggesting the Presence of Cystine in the Molecule

(chick embryo/tropocollagen/gel filtration)

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ABSTRACT When cells were isolated from chick-embryo tendons and incubated *in vitro* for 2–6 hr, essentially all the newly-synthesized collagen was recovered from the incubation medium as a transport form larger than tropocollagen. Experiments in which cells were incubated with [^{14}C]cystine suggested that the transport form contained cystine and that it was, in part, stabilized by disulfide bonds. Electron microscopy of segment-long-spacing aggregates prepared from the transport form of collagen showed that the native molecule differed from tropocollagen in that it had an extension of about 13 nm (130 Å) at the NH_2-terminal end.

Several laboratories have recently reported that cells *in vitro* synthesize polypeptide chains of collagen that are larger than the α-chains of interstitial collagen (1–8). The larger form recovered from the medium of fibroblasts in tissue culture (1, 2) had solubility properties that differed from those of α-chains in the native three-stranded conformation of tropocollagen. The polypeptide chains derived from the larger form of collagen had a different chromatographic behavior than α-chains but, after limited digestion with pepsin, they were apparently converted to α-chains (1, 2). A similar form of collagen was recovered from rat calvaria in organ culture (3, 7); it consisted of polypeptide chains with a molecular weight of 120,000 as estimated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (3). During the incubation of calvaria *in vitro*, there was a time-dependent conversion of the larger molecules to collagen, which was composed of α-chains (3, 7).

We have recently obtained matrix-free cells from embryonic chick tendon that synthesize collagen at a rapid rate for 2–6 hr *in vitro* (4, 5, 8). About 90% of the collagen synthesized by the cells is recovered in the incubation medium (4, 5), and all of it is composed of polypeptide chains with an apparent molecular weight of about 125,000, as estimated by gel filtration in an SDS–agarose column (8). However, if intact tendons are incubated under the same conditions, most of the collagen is converted to a form in which the polypeptide chains are of the same apparent size as α-chains, with a molecular weight of 95,000. These and other observations support the suggestion (2) that the larger form of collagen synthesized by cells is a transport form that is converted to tropocollagen after extrusion into the extracellular space of connective tissues.

Abbreviations: SDS, sodium dodecyl sulfate; SLS aggregates, segment-long-spacing aggregates.

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MATERIALS AND METHODS

Cells were isolated from the leg tendons of 17-day-old chick embryos by digestion of the tissue with trypsin and partially purified bacterial collagenase (4, 5, 8). The cells were incubated with [^{14}C]proline, 214 Ci/mol, [^{14}C]tryptophan, 6 Ci/mol, or [^{14}C]cystine, 271 Ci/mol (New England Nuclear Corp.), in 2 ml of Krebs medium at 37°C; the medium was removed by centrifugation at 1200 × *g* for 12 min.

For gel filtration, the medium from the cells was adjusted to 1% SDS (Sigma Chemical Co.), 1% mercaptoethanol, and 0.1 M sodium phosphate buffer (pH 7.4) by addition of concentrated stock solutions. The sample was incubated at 37°C for 3–18 hr, then dialyzed against 0.1% SDS in 0.1 M sodium phosphate buffer (pH 7.4) for 24 hr at room temperature. Gel filtration was at room temperature in a 1.5 × 90 cm column of 6% agarose (Bio-Gel A-5m, 200–400 mesh, Bio-Rad) equilibrated and eluted with 0.1% SDS–0.1 M sodium phosphate. The sample volume was 2.0 ml, the flow rate about 10 ml/hr, and the fraction size 2.0 ml. Aliquots of 0.2 or 0.4 ml were assayed directly for ^{14}C in a liquid scintillation counter (8), and the values shown are corrected for the size of the aliquots.

In order to prepare segment-long-spacing aggregates (SLS), the collagen in the medium was partially purified by the addition of solid ammonium sulfate (Baker Chemical Co.) to 30% saturation and centrifugation at 20,000 × *g* for 30 min. Precipitation and all further steps were done at 1–4°C. The pellet was dispersed in 1.0 ml of 0.1 N acetic acid and was dialyzed against 0.1 M Tris·HCl (pH 7.5)–0.4 M NaCl. The sample was centrifuged at 35,000 × *g* for 30 min to remove a nonradioactive precipitate, and the supernate was dialyzed overnight against 0.01 N acetic acid. The sample was then dialyzed against 0.1% disodium ATP (Sigma Chemical Co.) in 0.01 N acetic acid for 24 hr. One drop of the flocculent contents of the bag was placed on a carbon-coated grid and excess fluid was removed with a filter paper. The sample was then stained and examined in a Hitachi model HU-11-C electron microscope operated at 100 kV. Slide plates (Kodak contrast projector) were exposed to the image at a magnification of ×50,000.

For digestion with pepsin, 100 μg of the enzyme (Sigma) was added to 1.0 ml of a suspension of SLS aggregates, and

the sample was dialyzed at 4°C against 0.5 N acetic acid for 24 hr and at 18°C for 2 hr.

Medium was treated with tadpole collagenase by dialysis of 1.9 ml of medium against 0.4 M NaCl–0.1 M Tris·HCl, (pH 7.5); 0.1 ml of 0.1 M $CaCl_2$ was then added. The sample was then incubated at 20°C for 3 hr with 40 μg/ml of tadpole collagenase (a gift from Dr. Y. Nagai, Tokyo Medical and Dental University, who prepared it in highly purified form by affinity column chromatography). Bacterial collagenase treatment was done under the same conditions, except that 100 μg/ml of purified bacterial collagenase was added and the sample was incubated at 37°C for 90 min. The bacterial collagenase (Sigma Chemical Co.) was purified as described by Harper and Kang (9). Control experiments indicated that, under the conditions used here, neither the tadpole collagenase nor the bacterial collagenase hydrolyzed non-collagenous proteins prepared by incubation of chick-embryo livers with [^{14}C]tryptophan.

RESULTS

Evidence suggesting the presence of cystine in the molecule

90×10^6 cells isolated from chick-embryo tendons were incubated with 6 μCi of [^{14}C]proline in 18 ml of modified Krebs medium for 3 hr. The medium was removed from the cells by centrifugation and was dialyzed overnight against several changes of 0.4 M NaCl–0.1 M Tris·HCl (pH 7.5). An aliquot corresponding to one-thirtieth of the total sample was mixed with 2 mg of acid-soluble collagen from rat skin, and the combined sample was treated with 1% SDS–1% mercaptoethanol. Gel filtration of the combined sample on an SDS–agarose column (8) indicated that most of the protein-bound [^{14}C]proline eluted between the elution positions of the carrier α-chains and β-chains of collagen (Fig. 1). As reported (4, 5, 8), the [^{14}C]protein in the major peak was identified as polypeptide chains from a form of collagen by (*i*) its high ratio of [^{14}C]hydroxyproline to [^{14}C]proline, (*ii*) its digestion by bacterial collagenase, and (*iii*) the observation that if the [^{14}C]protein in its native state was subjected to limited digestion by pepsin before chromatography of SDS–agarose, the [^{14}C]proline-labeled polypeptides then eluted in the same position as α-chains.

Isolated tendon cells were incubated with ^{14}C-labeled cystine or tryptophan, two amino acids that are not found in the α-chains of interstitial collagens. When the cells were incubated with [^{14}C]tryptophan, all the incorporated ^{14}C was recovered in peptides considerably smaller than α-chains (Fig. 2). When the cells were incubated with [^{14}C]cystine, the incorporation of ^{14}C was only 3–6% of that obtained with [^{14}C]proline of about the same specific activity; about half of the incorporated [^{14}C]cystine was in small peptides (Fig. 2). However, a peak of ^{14}C appeared in the same position as did [^{14}C]proline-labeled polypeptides from the transport form. After medium from cells incubated with [^{14}C]-cystine was treated with tadpole collagenase, there was a marked decrease in the ^{14}C peak, which had previously eluted with the polypeptides from the transport form (Fig. 3). There was a proportional increase of ^{14}C that eluted later from the column; there was no decrease in the amount of nondialyzable ^{14}C. After the [^{14}C]cystine-labeled medium was treated with bacterial collagenase, the first peak of ^{14}C disappeared (Fig. 3). Treatment with bacterial collagenase decreased the recovery of nondialyzable ^{14}C by about 50%.

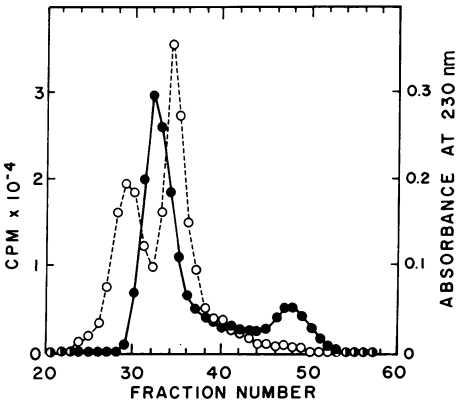

FIG. 1. Cochromatography on SDS–agarose of the medium from tendon cells incubated with [^{14}C]proline, and of acid-soluble collagen. The sample was treated with 1% SDS–1% mercaptoethanol. Acid-soluble collagen was purified from rat skin by extraction with acetic acid and precipitation with NaCl (10). Polyacrylamide gel electrophoresis (11) indicated that it consisted almost entirely of α- and β-chains, with only trace amounts of γ-chains and other components. Separate experiments with collagen purified by carboxymethyl-cellulose chromatography (12) indicated that the first peak of UV absorbance corresponded to β-chains, and the second peak to α-chains. The void volume of the column was in fraction 19, and the column volume was in fraction 61. The major peak of ^{14}C is somewhat broader than that obtained when the medium was chromatographed alone (compare with Fig. 2), probably because of trailing effects produced by the carrier collagen. Elution of ^{14}C (●—●); absorbance at 230 nm (○- - -○).

When the medium from cells incubated with [^{14}C]proline was treated with SDS, but not reduced with mercaptoethanol, there was a decrease in ^{14}C eluting with the transport form; an equivalent amount of ^{14}C appeared in a new peak, which eluted between the void volume and the elution position of β-chains (Fig. 4). There were no well-characterized proteins available with molecular weights greater than 250,000 that behaved as rod-like structures after denaturation with SDS; therefore, the column could not be calibrated with such proteins. However, extrapolation of the standard curve obtained with smaller proteins (8) suggested that the [^{14}C]-protein in the new peak had a molecular weight between 300,000 and 400,000. This estimate of size is highly approximate (see below), but it raises the possibility that the peak contained three covalently bound polypeptides from the transport form. In four separate experiments, the relative amount of ^{14}C in the new peak varied from 30 to 60% of the total [^{14}C]protein in the medium. A significant amount of ^{14}C eluted in the same position as the individual polypeptides of the transport form (Fig. 4).

When cells were incubated with [^{14}C]cystine and the medium was treated with SDS, but not with mercaptoethanol, a peak of ^{14}C appeared in the same elution position as the early peak seen when [^{14}C]proline was used as the source of label (Fig. 4). Although there was considerable variation from experiment to experiment in the relative amount of ^{14}C in the early peak (see above), in all the experiments the ratio of ^{14}C in the early peak to ^{14}C in the peak containing the individual polypeptides from the transport form was the same, whether the cells were incubated with [^{14}C]proline or [^{14}C]-cystine. Treatment of the medium with mercaptoethanol

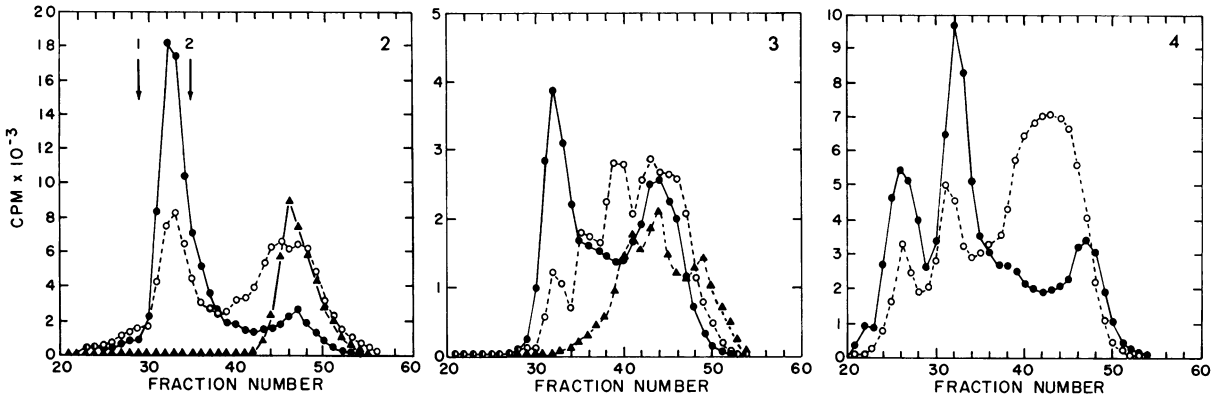

FIG. 2. Gel filtration on SDS–agarose of medium treated with 1% SDS–1% mercaptoethanol. Medium from 10^7 cells incubated for 4 hr with 1.5 μCi of [^{14}C]proline (●–●), for 4 hr with 15 μCi of [^{14}C]cystine (O- - -O), or for 2 hr with 8 μCi of [^{14}C]tryptophan (▲—▲). *Arrows* indicate the elution positions of β-chains [*1*] and α-chains [*2*].

FIG. 3. Effect of tadpole collagenase and bacterial collagenase on the elution pattern of medium on SDS–agarose. 2 × 10^7 cells were incubated for 4 hr with 30 μCi of [^{14}C]cystine. The medium was treated with tadpole collagenase or bacterial collagenase, then treated with 1% mercaptoethanol–1% SDS and dialyzed. Control sample of medium (●—●), medium treated with tadpole collagenase (O- - -O), medium treated with bacterial collagenase (▲—▲).

FIG. 4. Gel filtration on SDS–agarose of medium treated with 1% SDS, but not reduced with mercaptoethanol. Medium from 10^7 cells incubated for 4 hr with [^{14}C]proline (●——●) or for 4 hr with [^{14}C]cystine (O- - -O).

always produced a quantitative transfer of the ^{14}C from the early peak to the peak containing the individual polypeptides of the transport form (8), an observation that established that the early peak was composed of the same polypeptides.

Electron microscopy of SLS aggregates from the transport form

Quantitative assays for hydroxyproline (13) indicated that 30–50 μg of newly-synthesized collagen was recovered from the medium when 3 × 10^7 isolated tendon cells were incubated for 4–8 hr in either Krebs medium or Eagle's minimum essential medium with glutamine.

In order to prepare samples for electron microscopy, 2 × 10^8 cells were incubated with 6 μCi of [^{14}C]proline in Krebs medium for 6 hr so that the medium contained about 250 μg of newly-synthesized collagen. The collagen in the medium was partially purified by precipitation with ammonium sulfate; SLS aggregates were then prepared by the standard procedure used for interstitial collagen. Assays of the ^{14}C in the protein indicated that over 80% of the collagen in the medium was precipitated in the ammonium sulfate step, and that essentially all of the collagen was recovered by extraction of the pellet with acetic acid. After either negative or positive staining, the aggregates showed a cross-striated banding pattern, which is characteristic of SLS aggregates from tropocollagen. With negative stains, the end of the aggregates were sharply defined; with positive stains, the A- and B-ends were identifiable. The only difference between SLS aggregates from the medium of the cells and SLS aggregates from tendon was that all of the aggregates from the medium were about 130-Å longer (Fig. 5*a* and *b*), because of an extension at the A- or NH_2-terminal end.

Positive staining of the SLS aggregates with phosphotungstic acid or with uranyl acetate demonstrated the characteristic banding pattern in the tropocollagen part of the segments, but no distinct bands were seen in the extension at the A-end. The results suggested, therefore, that there was no clustering of positive or negative charges in the extension similar to that seen in the rest of the molecule.

Since limited pepsin digestion of the native transport-form reduced the apparent size of the polypeptide chains to that of α-chains (8), the preparation of SLS aggregates was treated with pepsin and SLS aggregates were again prepared. The treatment completely removed the extension at the A-end of all the aggregates (Fig. 5*c*), but no changes were observed at the B-end of the aggregates.

The native transport form was also treated with tadpole collagenase, then SLS aggregates were prepared (Fig. 5*d*). The enzyme cleaved the molecules at the b^2_2 band of the SLS aggregates, as was observed with tropocollagen (14). There was no change in the appearance of the extension at the A-end, an observation that confirmed the impression that the extension was an integral part of the NH_2-terminal end.

DISCUSSION

Essentially all the polypeptide chains in collagen synthesized by isolated tendon cells *in vitro* eluted ahead of α-chains when the collagen, treated with mercaptoethanol and SDS, was examined by gel filtration in SDS–agarose (8). The electron microscopy of SLS aggregates presented here demonstrates that all of this transport form of collagen contains an extension of about 130 Å at the A-end of the tropocollagen molecule. In all other respects, the aggregates are identical to SLS aggregates from interstitial collagen. Therefore, a major difference between the transport form and tropocollagen is the presence of extensions at the NH_2-terminal ends of each of the three α-chains.

If the NH_2-terminal extension on the transport form consists of amino acids in a triple-helical conformation similar to that in the rest of the tropocollagen molecule, the additional peptide on each α-chain would be about 5% of the size of the α-chain, or a molecular weight of about 5000. If, as seems more likely, the extension differs from the rest of the molecule, and has a conformation closer to that found in globular proteins, its size is more difficult to estimate. After the molecule is stained with ammonium molybdate, the extension has about the same diameter as the tropocollagen portion of the same

aggregate; therefore, its diameter appears to be no more than 15–20 Å. If we assume that the extension forms a cylindrical structure of 130 × 20 Å, and has a partial specific volume of 0.7, the additional molecular weight per α-chain would be about 12,000. The molecular weight estimate would be slightly higher if the extension contained sugars (with a partial specific volume of 0.5).

These calculations indicate that the maximal value for the size of the extension consistent with the electron micrographs is less than the value of about 30,000 molecular weight units per polypeptide chain that was suggested by gel filtration in SDS–agarose. The discrepancy may be explained by recent studies, which indicate that although many proteins become comparable rod-like structures when treated with SDS (15), some behave anomalously (16). The additional moieties on the polypeptides from the transport form might well have physical properties that are different from the rest of the molecule and that exaggerate the difference in apparent molecular weight from α-chains in SDS–agarose.

Experiments in which the isolated cells were incubated with [^{14}C]cystine indicated that the polypeptide chains of the transport form contained either cysteine or cystine. A significant fraction of the ^{14}C incorporated from [^{14}C]cystine eluted in the same position from the SDS–agarose column as did the polypeptide chains from the transport form of collagen. After treatment of the medium with a specific tadpole collagenase, there was a marked decrease in the [^{14}C]cystine-labeled protein that eluted in the same position as the collagen polypeptides; after treatment with a specific bacterial collagenase, the peak disappeared. Also, when reduction with mercaptoethanol before chromatography in SDS–agarose was omitted, part of the [^{14}C]proline-labeled protein shifted from the peak of collagen polypeptides to a new, rapidly eluting peak in the chromatogram. Under the same conditions, a proportional shift from the peak of collagen polypeptides to the new peak was observed with [^{14}C]cystine-labeled protein. The effect of mercaptoethanol further indicates that disulfide bonds help to hold together the polypeptide chains of the transport form. It was not possible to obtain direct evidence that the cysteine or cystine is present in the extensions in the NH_2-terminal ends, and significant amounts of the single polypeptide-chains of the transport form were recovered from the SDS-agarose column when the ^{14}C-labeled protein was not reduced with mercaptoethanol. Therefore, it was not clear whether all the polypeptide chains in the transport form are held together by disulfide bonds.

Most or all of the collagen extruded by isolated tendon cells is in a triple-helical conformation, since the tropocollagen portion of the molecule is resistant to digestion by pepsin (8). However, our preliminary observations indicate that the transport form does not aggregate into fibers under conditions that induce fiber formation from tropocollagen. The transport form of collagen may, therefore, be analogous to fibrinogen in that the formation of fibrin fibers is prevented until short peptides of 13–21 amino acids are cleaved from the NH_2-terminal ends of four of the six polypeptide chains found in fibrinogen (17).

It has recently been reported that in dermatosparaxis, a genetic disease of cattle, the skin is abnormally fragile and the collagen extracted from the skin contains cystine (18). Since the transport form of collagen apparently contains cystine, and since the NH_2-terminal extension is probably

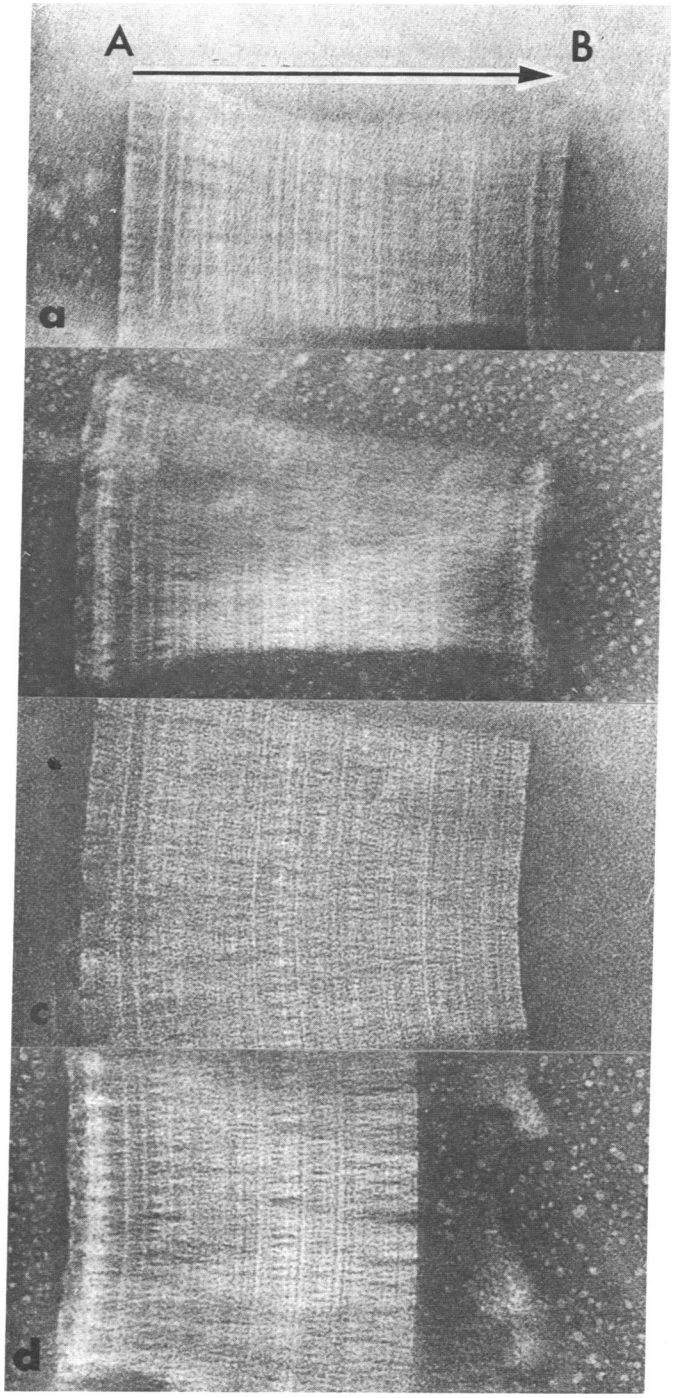

FIG. 5. (*a*). SLS aggregate formed by acid-soluble collagen extracted from the leg tendons of lathyritic chick embryos. The aggregate is negatively stained with 2% ammonium molybdate. (*b*). SLS aggregate from the transport form of collagen negatively stained with 4% ammonium molybdate. Note the presence of an extension at the A-end that is not seen in (*a*). (*c*). SLS aggregate from the transport form of collagen after pepsin treatment. The aggregate is negatively stained with 2% ammonium molybdate. Note the absence of an extension at the A-end. (*d*). SLS aggregate from the transport form of collagen after treatment with tadpole collagenase. The aggregate was negatively stained with 2% ammonium molybdate. The aggregate shown is the larger fragment obtained after cleavage by tadpole collagenase. The extension at the A-end is still attached. (*a*)–(*d*): × 200,000.

removed before the collagen forms interstitial fibers (8), we can speculate that dermatosparaxis may involve a defect in the conversion of the transport form to tropocollagen.

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