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Immunology

Purification and initial characterization of rat interleukin 2

(thymocyte stimulatory factor/T cell growth factor)

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ABSTRACT **With the sequential use of dialysis, chromatography on Sephadex G-100, reactive red 120-agarose, *p*-hydroxymercuribenzoate-agarose, phenyl-Sepharose, poly(L-lysine)-agarose, and isoelectrofocusing, the thymocyte stimulatory factor activity of interleukin 2 from rat spleen was purified about 8,000-fold. By the same procedures, the T cell growth factor activity of interleukin 2 was purified about 6,000-fold. The major peaks of thymocyte stimulatory factor activity and T cell growth factor activity cochromatographed in the various systems used, but T cell growth factor activity without significant thymocyte stimulatory factor activity was sometimes present. Both activities were found to have a pI between pH 5.50 and 6.30. Detectable thymocyte stimulatory factor activity was found at concentrations as low as 0.1–0.2 ng of protein per 0.2 ml. Dose–response plots were linear up to at least 1 ng of protein. Preparations of interleukin 2 labeled with ^{125}I-containing Bolton–Hunter reagent and purified by the procedure mentioned above were electrophoresed on a polyacrylamide gel under denaturing and reducing conditions. The ^{125}I-labeled material migrated in one major band with a molecular weight under 20,000 and three smaller bands with molecular weights of about 20,000, 60,000, and 90,000. Material with thymocyte stimulatory factor activity did not bind to a number of lectin-gels.**

Lymphokines involved in T cell–T cell interactions have been the object of intensive study in the past several years (1). The name interleukin 2 (Il2) has been coined (2) to indicate a substance (or family of substances) that: (*i*) confers to murine thymocytes the ability to respond to phytohemagglutinin (PHA) or concanavalin A (Con A) (3) and to participate in a mixed lymphocyte reaction (thymocyte stimulatory factor, TSF) (4); (*ii*) confers to thymocytes cytotoxic ability (5); (*iii*) induces helper activity in spleen cells of athymic mice (6); and (*iv*) is indispensable to sustain the growth of some cell lines (T cell growth factor, TCGF) (7). Considerable progress has been made in the past few years in defining some aspects of the biological activities of Il2, but it is clear that the elucidation of the relationships of these various activities among themselves and of the mechanism of action and biological significance of Il2 may be possible only if and when adequately pure material is available. The availability of an effective and relatively simple method for the purification of Il2 will also be of paramount importance for the study of the interrelationships between Il2 and other lymphokines. Di Sabato and coworkers (8, 9) have recently purified TSF from mixed lymphocyte cultures of murine spleen cells. The degree of purification, however, achieved in this and other work (10, 11) was relatively modest (300- to 400-fold). The present paper reports a substantial purification of the TSF and TCGF activities of Il2 from rat spleen. A preliminary characterization of this factor is also reported.

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MATERIALS AND METHODS

Preparation of Il2-Containing Supernatants (Stage I Cultures). Il2 from spleen cells of male Wistar rats weighing about 200 g was prepared in batches of 1–2 liters in Petri dishes containing RPMI 1640 medium supplemented with 2 mM glutamine and 0.1 mM alanine, 0.05 mM 2-mercaptoethanol, Con A at 5 μg/ml, and 3×10^8 cells. The total volume was 30 ml. No serum was present. After 2 days of incubation at 37°C in an atmosphere of 95% air and 5% CO_2, the cells were removed by centrifugation, and the supernatants were lyophilized and stored at −20°C until used.

Determination of Proteins. Proteins were labeled with L-[^{35}S]methionine by adding the labeled amino acid to stage I cultures prepared with methionineless medium. In other experiments, the supernatants of stage I cultures were lyophilized, passed through a Sephadex G-100 column, and labeled with Na^{125}I (12) or with ^{125}I-containing Bolton–Hunter reagent (New England Nuclear) (13). The excess label was removed by exhaustive dialysis. The procedure used for the absolute quantification of the proteins present at the various stages of purification was described in a previous paper (9).

Assay of TSF (Stage II Cultures). (See ref. 4.) The TSF activity of Il2 was assayed in Linbro microplates using 0.5×10^6 murine thymocytes per microwell in the presence of 0.8 μg of PHA and 10% pooled human serum. At 48 hr of incubation, 1 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]thymidine was added to each well. The cells were harvested 24 hr later and the amount of radioactivity incorporated was determined in a scintillation counter.

Assay of TCGF Activity. The TCGF activity was determined according to Gillis *et al.* (14) with only minor modifications. Briefly, 1×10^4 cytotoxic T lymphocyte line (CTLL) cells (grown in the presence of TCGF and 10% fetal calf serum to a density of about 0.5×10^6 cells per ml and exhaustively washed with RPMI 1640 medium before use) were incubated in Linbro microplates in the presence of RPMI 1640 medium, 10% fetal calf serum, 0.02 mM 2-mercaptoethanol, and 0.005% each of cytosine, uridine, adenosine, and guanosine. [^{3}H]Thymidine (1 μCi) was also added. The total volume per well was 0.2 ml. After 24 hr of incubation at 37°C in an atmosphere of 95% air and 5% CO_2, the cells were harvested and the radioactivity incorporated in the CTLL was determined in a scintillation counter.

Samples were assayed for TSF and TCGF at least at two and, in most cases, at four different concentrations. Dose–response plots were linear in most cases.

Dialysis and Gel Filtration on Sephadex G-100. The lyophilized stage I culture supernatants were taken up in about

Abbreviations: Il2, interleukin 2; PHA, phytohemagglutinin; Con A, concanavalin A; TSF, thymocyte stimulatory factor; TCGF, T cell growth factor; PHMB, *p*-hydroxymercuribenzoate; CTLL, cytotoxic T lymphocyte line.

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1/30th of the original volume and dialyzed exhaustively vs. 0.02 M Tris·HCl buffer, pH 7.20. A precipitate formed and was removed by centrifugation. After lyophilization, the sample was taken up in 2–3 ml of water and applied to a Sephadex G-100 column (2.5 × 35 cm), equilibrated and eluted with 0.02 M Tris·HCl buffer, pH 7.20. Four-milliliter fractions were collected.

Chromatography on Red-Agarose (Reactive Red 120-Agarose). The Sephadex fractions containing TSF activity were pooled. An aliquot of the pool (usually 5 ml) was diluted to bring the protein concentration to about 0.15 mg/ml. The pH of the sample was brought to 5.90 by the addition of an appropriate amount of 1 M NaH_2PO_4. Phenylmethylsulfonyl fluoride (0.1 mM) was also present. The sample was then applied to a reactive red 120-agarose (Sigma) column (1 × 6 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 5.90. The column was eluted in sequence with: 0.05 M sodium phosphate buffer, pH 5.90 (containing 0.1 mM phenylmethylsulfonyl fluoride); a gradient from 0.05 M sodium phosphate buffer, pH 5.90, to 0.05 M Na_2HPO_4; 0.05 M NaH_2PO_4; and, finally, 1% sodium dodecyl sulfate. Five-milliliter fractions were collected; 0.2 ml of 1% bovine serum albumin (called "albumin" hereafter) was added to the fractions known to contain TSF activity. All fractions were dialyzed vs. 0.02 M Tris·HCl buffer, pH 7.20, before assay.

Chromatography on *p*-Hydroxymercuribenzoate (PHMB)-Agarose. The red-agarose fractions eluted with Na_2HPO_4 and containing TSF activity were pooled and applied to a PHMB-agarose (Sigma) column (0.8 × 2.5 cm) equilibrated and eluted up to tube 12 with 0.02 M Tris·HCl buffer, pH 7.20, containing 0.1% albumin. The elution was continued with 0.2 M cysteine in 0.02 M Tris·HCl buffer, pH 7.20, containing 0.1% albumin up to tube 20. In some experiments, after Tris buffer/0.1% albumin, the column was eluted for 8 tubes with 0.2 M NaCl in 0.02 M Tris·HCl buffer, pH 7.20, and 0.1% albumin. The elution was then continued with 0.2 M cysteine/0.1% albumin, as indicated above. Three-milliliter fractions were collected. The fractions eluted with cysteine or NaCl were dialyzed exhaustively vs. 0.02 M Tris·HCl buffer, pH 7.20, before assay.

Chromatography on Phenyl-Sepharose. The PHMB-agarose fractions eluted with Tris buffer/albumin and containing TSF activity were pooled and applied to a phenyl-Sepharose (Pharmacia) column (0.8 × 2.5 cm) equilibrated and eluted up to tube 12 with 0.02 M Tris·HCl buffer, pH 7.20/0.1% albumin. The elution was continued with 1 M NaCl in Tris buffer/0.1% albumin up to tube 20. Three-milliliter fractions were collected. The fractions eluted with 1 M NaCl were dialyzed exhaustively vs. 0.02 M Tris·HCl buffer, pH 7.20, before assay.

Chromatography on Poly(L-lysine)-Agarose. The fractions eluted from the phenyl-Sepharose column with Tris buffer/albumin and containing TSF activity were pooled and applied to a poly(L-lysine)-agarose (Sigma) column (0.8 × 2.5 cm) equilibrated and eluted up to tube 12 with 0.02 M Tris·HCl buffer, pH 7.20/0.1% albumin. The elution was continued up to tube 20 with 1 M NaCl in 0.02 M Tris·HCl buffer, pH 7.20/0.1% albumin. Three-milliliter fractions were collected. The fractions eluted with NaCl were dialyzed exhaustively vs. 0.02 M Tris·HCl buffer, pH 7.20, before assay.

Column Electrofocusing. Electrofocusing was carried out in an LKB apparatus according to the recommendations of the manufacturer. Sucrose gradients and Ampholine buffers in the range pH 3–10 were used. For further details, see ref. 9.

Electrophoresis on Polyacrylamide Gel. Electrofocused fractions of Il2 labeled with ^{125}I-containing Bolton–Hunter reagent were lyophilized and taken up in 0.05 ml of water to which 0.02 ml of a solution containing 4% sodium dodecyl sulfate, 20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and 2% (vol/vol) Triton X-100 in 0.125 M Tris·HCl buffer, pH 6.80, had been added. The material was electrophoresed for 4 hr in a 9% polyacrylamide gel containing 1% sodium dodecyl sulfate. The gel was then dried and cut into 0.2-cm slices, the radioactivity of which was determined in a Packard gamma counter.

Chromatography on Lectin-Gels. In these experiments, 1.0–1.5 ml of samples at different stages of purification were made 0.2 M in NaCl and applied to small columns (0.8 × 2 cm) of lectin-gel. The columns were equilibrated and eluted up to tube 12 with 0.02 M Tris·HCl buffer, pH 7.2, containing 0.2 M NaCl (0.2 M $CaCl_2$ for *Limulus polyphemus* lectin-agarose) and 0.1% albumin. The elution was then continued up to tube 20 with a solution of the specific carbohydrate in Tris buffer and 0.1% albumin: 0.2 M α-methyl mannoside (for Con A-agarose and lentil lectin-agarose), 0.2 M L-fucose (for *Lotus tetragonolobus* lectin-agarose), 0.1 M *N*-acetylgalactosamine (for *Helix pomatia* lectin-agarose), 0.2 M *N*-acetylglucosamine (for wheat germ lectin-agarose) and 0.01 M sialic acid or 0.2 M citric acid (for *Limulus polyphemus* lectin-agarose). Three-milliliter fractions were collected.

RESULTS

Dialysis at pH 7.20 and Gel Filtration on Sephadex G-100. The use of larger-scale preparations of Il2 compared to previous work (9) necessitated the removal by dialysis of the low molecular weight substances present in the lyophilized material before applying the sample to Sephadex columns. Although the purification achieved was modest, dialysis was effective in precipitating proteins that cochromatograph with Il2 on red-agarose. Similarly to what was found with murine Il2 (4, 9, 15), the activity was eluted from Sephadex G-100 in a broad band centered at about 30,000 daltons and sometimes presenting minor peaks.

Chromatography on Red-Agarose. As shown in Fig. 1, most of the TSF and TCGF activity and some protein were eluted from the red-agarose column with 0.05 M Na_2HPO_4. The bulk of the proteins were eluted with 1% sodium dodecyl sulfate. The purification achieved with this step was about 13-fold. Greater than 70% of the TSF and TCGF activity and 80% of the proteins applied on the column were recovered.

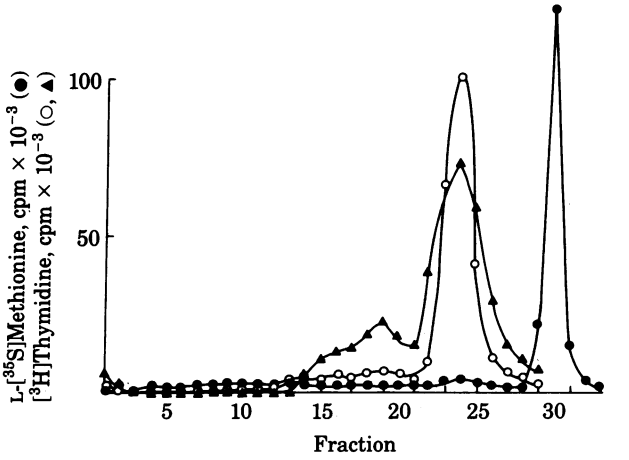

FIG. 1. Chromatography of Il2 on red-agarose. The column was eluted with 0.05 M sodium phosphate buffer, pH 5.9, up to tube 12, with a gradient from 0.05 M sodium phosphate buffer, pH 5.90, to 0.05 M Na_2HPO_4 from tube 12 to tube 20, with 0.05 M Na_2HPO_4 from tube 21 to tube 28, and with 1% sodium dodecyl sulfate from tube 29 to tube 33. ●, L-[^{35}S]Methionine-labeled proteins; ○, TSF activity; ▲, TCGF activity.

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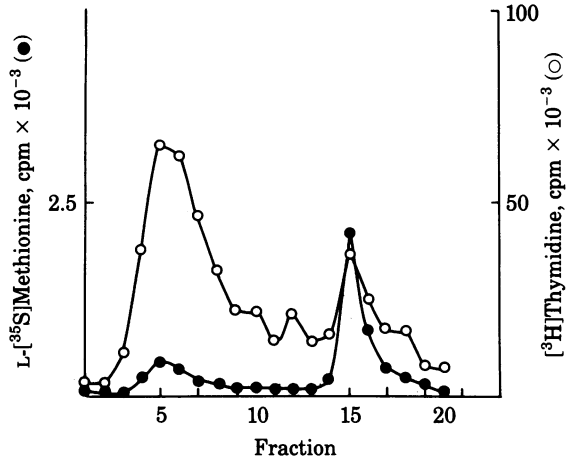

FIG. 2. Chromatography of Il2 on PHMB-agarose. The column was eluted with 0.02 M Tris·HCl buffer, pH 7.2/0.1% albumin up to tube 12 and with 0.2 M cysteine in the same buffer from tube 13 to tube 20. ●, L-[^{35}S]Methionine-labeled proteins; ○, TSF activity.

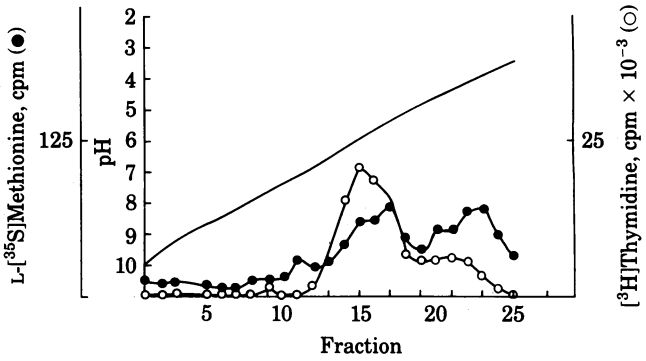

FIG. 3. Isoelectrofocusing of Il2. ——, pH; ●, L-[^{35}S]methionine-labeled proteins; ○, TSF activity.

Chromatography on PHMB-Agarose. The material purified on red-agarose was applied to a PHMB-agarose column. As shown in Fig. 2, about 80% of the TSF activity applied to the column and about 0.1% of the original proteins were eluted with Tris buffer. The purification achieved with this step was about 7-fold for the TSF activity and 4-fold for the TCGF activity. About 10–15% of the activity applied to the column was eluted with 0.2 M cysteine. About half of this activity, however, could be eluted with 0.2 M NaCl (see *Materials and Methods*). Incubation of the dialyzed cysteine-eluted material with 1 mM PHMB for 10 min at room temperature, followed by exhaustive dialysis vs. 0.02 M Tris·HCl buffer, pH 7.20, caused no detectable loss of activity. Part of this activity is probably not due to Il2, because the effluents of blank PHMB-agarose columns eluted with 0.2 M cysteine had some mitogenicity for thymocytes.

Chromatography on Phenyl-Sepharose. The fractions containing TSF activity eluted from the PHMB-agarose column with Tris buffer were pooled and applied to a phenyl-Sepharose column. Most of the activity applied was recovered in the Tris eluate. The overall purification achieved after this step was about 2-fold for both the TSF and the TCGF activities.

Chromatography on Poly(L-lysine)-Agarose. The material purified on phenyl-Sepharose was applied to a poly(L-lysine)-agarose column. Virtually all of the activity applied was recovered in the Tris eluate. Although this step did not greatly contribute to the purification of Il2, it was found to improve the efficiency of removal of contaminating proteins by isoelectrofocusing.

Isoelectrofocusing. The TSF-containing material eluted from the poly(L-lysine)-agarose column was electrofocused on a sucrose gradient (Fig. 3). Most of the TSF and TCGF activity focused in a rather broad band between pH 5.50 and 6.30. A minor (usually less than 15% of the total) peak of activity was present occasionally at pH 4.30–4.60. After this step the overall purification of the TSF activity was about 8,000-fold. The TCGF activity was purified about 6,000-fold. In general the main peaks of the two activities eluted in the various chromatographic systems coincided. Some fractions, however, showed significant TCGF activity with little or no TSF activity (e.g., Fig. 1).

In Table 1, the recovery of TSF and TCGF activities, the recovery of protein (measured as L-[^{35}S]methionine), and the purification factor attained at each step of the purification are shown.

Experiments with ^{125}I and Gel Electrophoresis. In some experiments the proteins present in the supernatant of the stage I cultures were labeled with Na^{125}I (see *Materials and Methods*). The ^{125}I-labeled preparations were purified by the procedure described above for the L-[^{35}S]methionine-labeled material. The recovery of ^{125}I-labeled proteins at each step was similar to that with L-[^{35}S]methionine-labeled materials, indicating that the L-[^{35}S]methionine radioactivity is an accurate measure of the proteins present in the preparation. In other experiments, Il2 labeled with ^{125}I-containing Bolton–Hunter reagent was purified as described in previous paragraphs. After electrofocusing the purification factor of a typical preparation was 7,550-fold. The electrofocused fractions containing Il2 activity (pI 5.50–6.30) were pooled, lyophilized, and electrophoresed as detailed in *Materials and Methods*. Fig. 4 shows that the ^{125}I-labeled material migrates as one major band with a molecular weight under 20,000 and three smaller bands with molecular weights of about 20,000, 60,000, and 90,000.

Activity of Purified Il2. Isoelectrofocusing-purified preparations of Il2 (pI 5.50–6.30 fractions) showed detectable TSF activity at concentrations of protein as low as 0.1–0.2 ng/0.2 ml. Dose–response plots were linear at least up to 1 ng of protein

Table 1. Purification of TSF and TCGF activities of Il2

Step	Total protein, mg	Recovery, %		Purification factor	
		TSF	TCGF	TSF	TCGF
Crude	8	100	100	1	1
Dialysis	5	100	100	1.6	1.6
Sephadex G-100	1.6	97	95	4.9	4.8
Red-agarose	0.088	71	66	64	60
PHMB-agarose	0.010	54	30	432	240
Phenyl-Sepharose	0.0044	43	27	780	491
Poly(L-lysine)-agarose	0.0040	44	29	881	579
Isoelectrofocusing*	0.00012	12	9	8,003	6,001

* Includes only fraction with pI 5.50–6.30.

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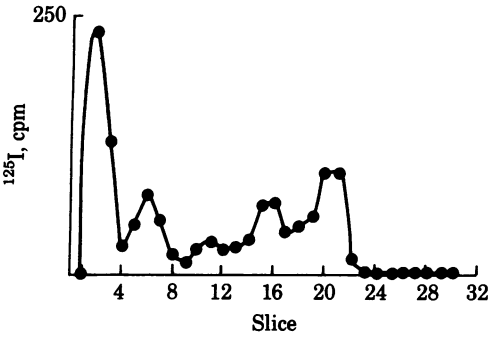

FIG. 4. Gel electrophoresis of purified Il2. Slices 6, 16, and 20 correspond to material with molecular weight of 20,000, 60,000, and 90,000, respectively.

per 0.2 ml. No mitogenic activity was detected in the absence of PHA.

Chromatography on Lectin-Gels. In an effort to establish whether Il2 is a glycoprotein, preparations purified on red-agarose, PHMB-agarose, and isoelectrofocusing were applied to a number of lectin-gel columns (see *Materials and Methods*). In every case, more than 95% of the applied TSF activity was recovered in the Tris buffer eluate. Only insignificant amounts were eluted with the specific carbohydrate.

DISCUSSION

Various procedures (16, 17), including the unsuccessful use of immuno-affinity chromatography with monoclonal antibodies (18), have been applied to the purification of Il2 from different sources (19). It is difficult, however, to assess the degree of purification achieved in most of this work, because no data on the putative increase of specific activity of the preparations are reported. The present work reports an 8,000-fold purification of TSF activity and 6,000-fold purification of TCGF activity of Il2 from rat spleen cells. To our knowledge, this is the highest degree of purification of an interleukin and one of the highest obtained for a lymphokine, including interferon. This was achieved by the sequential use of dialysis, gel chromatography on Sephadex G-100, red-agarose, PHMB-agarose, phenyl-Sepharose, and poly(L-lysine)-agarose, and isoelectrofocusing. The active material focused with pI of 5.50–6.30, clearly different from the pI of purified murine TSF (4.70–5.10) (9). The minor peak of activity appearing at pH 4.30–4.60 is probably expression of a certain degree of molecular heterogeneity present in interleukins (e.g., see ref. 9). Critical to the achievement of a high degree of purification was the addition of albumin to the elution buffers. Without added protein, the recoveries of activity beyond the red-agarose step were unacceptably low (less than 10–15%). It should be clear, however, that the purification factor does not take into account the added albumin, but only the proteins produced, and biosynthetically labeled with L-[^{35}S]methionine, in the stage I cultures. Appropriate controls firmly established that albumin, at the concentrations used in this work, did not affect the TSF and TCGF assays.

In the present work both TSF and TCGF activities were purified. The fact that the main peaks of these activities cochromatograph in all the various systems used and that they purify to a similar extent indicates that both activities are due to the same substance. In some fractions, however, TCGF activity with no detectable or negligible TSF activity was present. A similar observation has been reported by Evinger *et al.* (20) for the anti-viral and growth-inhibitory activity of interferon. In the interpretation of this phenomenon, the possibility that different cell populations may have different degrees of responsiveness to the various molecular forms of Il2 should be kept in mind.

The purest preparations of the Il2 obtained in this work show clear TSF activity at a protein concentration as low as 0.1 ng/0.2 ml in the presence of 5×10^5 thymocytes. Thus, assuming a molecular weight of 30,000 (4), Il2 is active at about 10 pM.

Purified preparations of Il2 electrophoresed under denaturing and reducing conditions show one major band of material with molecular weight under 20,000 and three smaller bands with molecular weights of about 20,000, 60,000, and 90,000. Some of this material is probably contaminating proteins still present in the preparation; the material with molecular weight under 20,000 may be the molecular weight 16,000 subunit obtained by Caplan *et al.* (21) upon treatment of murine Il2 with sodium dodecyl sulfate.

The failure to bind significant amounts of TSF to lectin-gels indicates either that rat Il2 is not a glycoprotein or, if carbohydrate residues are present, that they are not readily accessible. From this point of view, rat Il2 is different from mouse Il2, which binds to Con A-Sepharose (9).

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