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Characterization of a tryptic digest by high-performance displacement chromatography and mass spectrometry[a]

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ABSTRACT

High-performance displacement chromatography (HPDC) provides a means of increasing the capacity of a chromatographic column, while maintaining the resolution afforded by high-performance liquid chromatographic (HPLC) instruments. The high capacity and high resolution of HPDC can be exploited in tryptic mapping to facilitate the characterization of a protein preparation. In this manner, minor constituents of the mixture, which may be difficult to isolate by conventional chromatographic methods, can be obtained in sufficient amounts to permit chemical characterization by established techniques. The isolation by HPDC of peptides obtained by digestion of recombinant human growth hormone (rhGH) and the subsequent characterization of the peptides are described. The identification of certain of these peptides revealed information on the specificity of trypsin for the substrate, rhGH, and for autolysis. Fractions from the HPDC tryptic map were collected and analyzed by electrospray ionization mass spectrometry (ESI-MS) either directly or following further separation by gradient elution HPLC. Fragment ions observed in the ESI mass spectra facilitated identification of peptides obtained by HPDC tryptic mapping.

INTRODUCTION

Displacement chromatography is a mode of operating a chromatographic separation that significantly increases the feed loading capacity of the column while maintaining high resolution and high speed. The enhanced capacity attainable in displacement chromatography has prompted studies of its feasibility for preparative- and process-scale purification [1–6]. While the main features of displacement chromatography were described by Tiselius almost 50 years ago [7], it has recently been re-established as a convenient means of increasing the capacity of preparative high-

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performance liquid chromatography (HPLC). The resulting approach to preparative separations on high-efficiency analytical instrumentation has been termed high-performance displacement chromatography (HPDC) [8,9]. This approach has been shown to increase by one to two orders of magnitude the amount of a feed mixture that can be purified in a single run on analytical chromatographic equipment [10–12].

The feature that distinguishes displacement from elution chromatography is that rather than being washed (eluted) from the column, the feed components are displaced from the surface by a solution of a strongly retained substance that follows the feed into the column. In elution chromatography the eluent contains a modifier, whether an organic solvent in reversed-phase chromatography or a salt in ion-exchange chromatography, that is less strongly retained than the feed components to be separated. The modifier is employed at a sufficiently high concentration that it washes the feed components from the surface and as a result the separated bands of the feed mixture leave the column mixed with the eluent modifier. In displacement chromatography, on the other hand, the displacer is more strongly retained than the feed components, it is pumped into the column only after the feed mixture has been loaded and the separated bands leave the column just *ahead* of the displacer, rather than mixed with it. As described more fully below, the separated feed components leave the column as adjacent, rectangular bands, rather than as discrete peaks as in elution chromatography. The concentrations of the bands containing individual purified feed components are fixed, independent of the amount of the feed [13], so the volume occupied by a component as it leaves the column is uncoupled from its resolution from other feed components, unlike peak width in elution chromatography. In the latter instance, resolution suffers as the peak width and loading increase [14], so the column capacity is governed by the band-broadening characteristics of the separation conditions. The added control over the concentration and volume of the separated bands afforded by operating the column in the displacement mode increases the capacity of the chromatographic equipment and accounts for the advantages of this approach for large-scale, high-performance separations [15].

The high capacity and control over the concentrations of bands in displacement chromatography also have potential advantages for enhancing the sensitivity of analytical and micro-preparative applications of HPDC. One factor determining the detectability of a component of a mixture is the amount of that component in the sample, so increasing the feed capacity in an analytical system increases the detectability of minor components of a complex mixture. In addition, as noted above, the concentration of a band in displacement chromatography is controlled by the operating conditions, so even a minor component will be focused into a narrow, concentrated band in the fully developed displacement chromatogram. This focusing effect can be exploited to enhance significantly the detectability of trace mixture components, as has been argued by Guiochon and co-workers [16,17].

The displacement mode has previously been applied to the analysis of a tryptic mixture by capillary liquid chromatography coupled with continuous-flow fast atom bombardment mass spectrometry [18]. In that application, the flow-rate and column dimensions were constrained by the high-vacuum requirements of the mass spectrometer, so HPDC permitted enhanced column capacity and increased detectability of minor components of the mixture. Those findings also revealed that the mass spectrometer is a nearly ideal detector for displacement chromatography of peptides as it

rapidly and selectively monitors the composition of the displacement train leaving the column.

The high column capacity and focusing of minor components of a mixture can be applied to effect the preparative isolation of useable amounts of minor components of a mixture for characterization by a variety of techniques. In this paper, the isolation of components of a tryptic digest of recombinant human growth hormone (rhGH) is demonstrated on conventional analytical-scale columns and instrumentation. Off-line approaches to coupled displacement chromatography and mass spectrometry and sequential displacement chromatography with separation of fractions by elution liquid chromatography and identification of peptides by mass spectrometry were used to confirm the identity of peptides identified previously by directly coupled DC–MS [18]. The coupling of DC with LC analysis of fractions was employed to provide quantitative assessment of the amounts of peptides identified by MS. Fragmentation information has been obtained from spectra of peptides analyzed on an electrospray ionization mass spectrometer. This additional information confirmed the amino acid sequence for certain of the peptide molecular ions [19,20].

THEORY

Displacement chromatography, as described originally by Tiselius [7], is the mode of chromatography in which a solution of a substance, the displacer, with a stronger affinity for the stationary phase than any of the feed components, is made to flow into the column behind the feed. The operating conditions are chosen so that the displacer saturates the column and drives the feed mixture ahead of it. The concentration of the displacer is constant at the column inlet, so the saturation front moves with constant velocity as a step increase in concentration down the column. Ahead of the displacer, the feed components arrange themselves into rectangular bands that move together, in sequence, according to their relative affinities for the stationary phase, in a "displacement train" down the column. Despite the similarities in terminology, displacement chromatography is distinct from affinity chromatography [21], as no specific interaction between a feed component and a bound ligand is exploited in HPDC. Rather, *relative* affinities among the feed components and displacer are exploited to achieve separation, and the HPDC operating mode can be carried out on non-specific sorbents such as those used in reversed-phase and ion-exchange chromatography.

Feed components arrange themselves into rectangular bands in the displacement train under the influence of the displacer front, which moves the feed mixture forward at constant velocity, and the adsorption isotherms that govern the equilibrium between the mobile and stationary phases. In many adsorptive processes the adsorption isotherms for individual components are concave downward, such as are shown in Fig. 1 for four different species. The concave downward isotherm is characteristic of a saturable stationary phase surface. The adsorption isotherm governs the phase equilibrium of a substance and, concomitantly, governs the velocity of the substance in the chromatographic column. The speed of the displacer front as it moves down the column is given by the expression [22]

$$v_d = \frac{v_o}{1 + \Phi q_d / c_d} \tag{1}$$

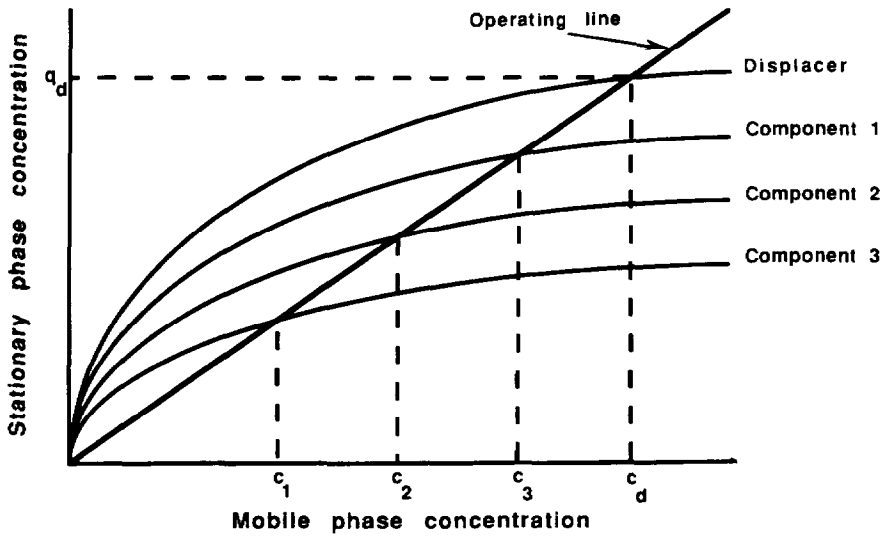

Fig. 1. Schematic diagram of individual adsorption isotherms illustrating the relationships among feed component concentrations and the displacer concentration in the displacement train. The operating line connects the point on the isotherm corresponding to the displacer concentration, c_d, with the origin. c_1, c_2 and c_3 are the isotachic concentrations of the feed components and c_d is the displacer concentration.

where v_0 is the velocity of the mobile phase through the column, q_d is the stationary phase concentration of the displacer in equilibrium with a mobile phase concentration c_d and Φ is the phase ratio of the column. The ratio $q_d c_d$ which, from eqn. 1, determines the speed at which the displacer moves down the column, can be represented graphically by the chord to the displacer isotherm in Fig. 1. In the fully developed displacement train, each separated component of the feed moves at the same speed as the displacer front, and the velocity of the individual bands in the train can be described by equations exactly analogous to eqn. 1. Hence, the velocities of the individual species in the train are also governed by the ratio q_i/c_i, where i represents a single component of the feed mixture. As the velocities of all components of the mixture are equal to that of the displacer front in the fully developed train, then

$$q_d/c_d = q_i/c_i \tag{2}$$

for all components of the mixture. Graphically, this is represented in Fig. 1 by the intersection of the chord to the displacer concentration with each individual isotherm of the feed components. The chord thus governs the speed of the displacer front and of the bands in the displacement train, and has been termed the "operating line" [23]. The point at which the operating line intersects its isotherm governs the speed of the zone of an individual component through the column and fixes the concentration of the zone in the displacement train. This accounts for the rectangular bands characteristic of displacement chromatography, as individual components are thermodynamically contrained to move through the column at the concentration established by the relationship in eqn. 2. As the concentration of a band in the displacement train is

fixed, the volume occupied by a given component of the mixture is variable, and depends on the amount of that component present in the feed mixture. Hence a major constituent of the mixture will form a relatively wide band in the displacement train whereas a trace component of the mixture will form a relatively narrow band [16,17].

These features account for certain of the advantages of the displacement mode over conventional elution chromatography in both preparative and analytical separations. In elution chromatography, the relationship between band height and width (*i.e.*, a solute's concentration and volume) is more complex than in HPDC. When the column is underloaded, all peaks have roughly the same width, so that peak height is dependent on the amount of a component present in the feed mixture and retention times are constant. This simple, predictable relationship accounts for the broad acceptance of linear elution chromatography since its inception 40 year ago [24]. On overloading the column, the peaks of the major constituents of the mixture will be overloaded, with both high concentrations at the apex and broadening due to the skewing effect of saturating the stationary phase [22]. Minor components of the mixture, on the other hand, form small peaks, which may also be skewed owing to interference effects from the major components of the mixture [25]. The complex behavior of overloaded elution chromatography limits its utility in analytical applications.

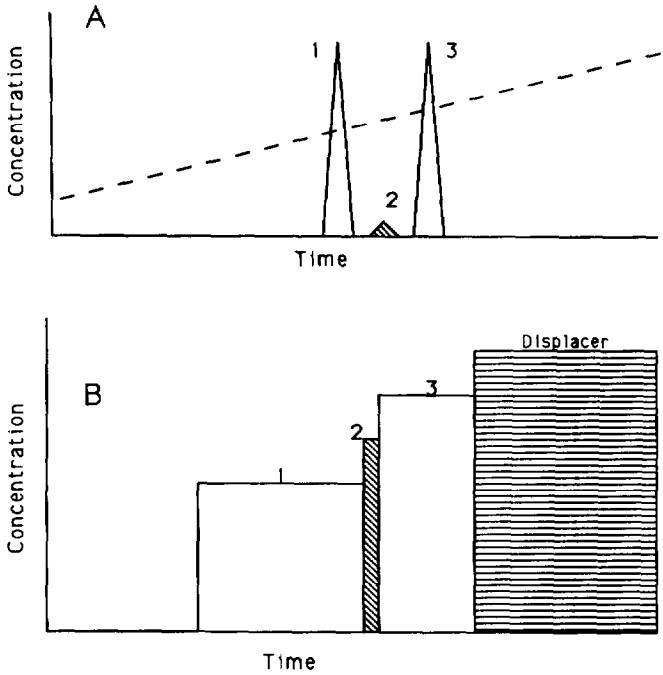

Fig. 2. Comparison of (A) elution and (B) displacement chromatograms of a mixture consisting of two major components (1 and 3) and one trace component (2). The dashed line in (A) illustrates the increase in eluent modifier concentration in the mobile phase in gradient elution chromatography. The larger feed load and focusing effect afforded by operating in the displacement mode are illustrated in (B), and account for the advantages of HPDC for the analysis of minor components of a mixture.

The analysis of minor components of a mixture can be facilitated by using the displacement mode of chromatography, in which all bands within the displacement train form relatively concentrated bands, as described above. The minor components are thus focused between bands of major components of the mixture, as shown schematically in Fig. 2, which compares chromatograms obtained in linear elution and displacement chromatography. The linear elution profile (Fig. 2A) shows the minor component of the mixture as a small peak between two larger peaks that represent major components. In the displacement chromatogram (Fig. 2B), the minor component is represented as a tall narrow band focused between two larger, broad rectangular bands of the major components of the mixture. Fig. 2 illustrates the two advantages expected from analytical displacement chromatography: first, the minor components of the mixture are focused into narrow bands, and second, a larger feed can be separated in a single run, permitting a larger loading of trace components. The larger load and focusing of minor components by displacement chromatography have been advanced as a powerful means of enhancing the detectability of these species [16–18].

EXPERIMENTAL

Materials

Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, USA). L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated bovine trypsin was obtained from Worthington (Freehold, NJ, USA). Water was purified with a Milli-Q system from Millipore (Bedford, MA, USA). Hydrochloric acid and cetyltrimethylammonium bromide (CTAB) were from Aldrich (St. Louis, MO, USA) and HPLC-grade acetonitrile (ACN) from Burdick & Jackson (Muskegon, MI, USA). Recombinant human growth hormone was expressed in *E. coli* at Genentech. All other chemicals were of ACS grade or better.

Methods

Tryptic digest. Digestion with trypsin was carried out by reconstituting 10 mg of rhGH in 4 ml of 100 m*M* Tris–acetate buffer (pH 8.3), warming the sample to 37°C and adding 100 μg of trypsin. After 2 h, a second aliquot of 100 μg of trypsin was added. Digestion was stopped 2 h later by lowering the pH of the solution below 3 with 1 *M* hydrochloric acid. The digest mixture was stored at 5°C until analyzed.

HPLC analyses. Tryptic maps were produced by reversed-phase HPLC on a Hewlett-Packard (Palo Alto, CA, USA) Model 1090M system equipped with a Nucleosil C_{18} (Alltech, Deerfield, IL, USA) column. An aliquot of the tryptic digest mixture was injected into the column equilibrated at 35°C with 0.1% aqueous TFA at a flow-rate of 1 ml/min. A linear gradient to 40% ACN over 20 min was started upon injection. At the end of the gradient, the ACN content was increased to 70% over 1 min. The column effluent was monitored at 214 nm. Peaks were identified by comparison of retention times with previously characterized maps [26]. Analyses of fractions collected from the displacement separation were carried out under the same conditions. Peaks analyzed off-line by mass spectrometry were collected manually from separations carried out under identical conditions, except on an HP 1050 instrument equipped with a FIatron Laboratory Systems (Oconomowoc, WI, USA) Model CH-30 column heater.

HPDC tryptic map. The displacement separations were carried out on the Hewlett-Packard 1090M HPLC instrument with two 150 mm × 0.46 mm (I.D.) Nucleosil C_{18} columns connected in series. A 9-mg aliquot of the digest mixture was injected into the column, equilibrated with a carrier solvent consisting of 0.1% TFA in water. After a 5-min hold, the displacer solution consisting of CTAB and 0.1% TFA in water was pumped into the column at a flow-rate of 1 ml/min. The switch from starting eluent to displacer solution occurred within a 1-min period. Fractions were collected at 1-min intervals. The absorbance detector monitored the column effluent at 214, 254 and 280 nm.

Mass spectrometry. Mass spectra were acquired with a Sciex (Thornhill, ON, Canada) API-III triple quadrupole mass spectrometer equipped with an ionspray interface. Peptides were infused in a solution of 50% acetonitrile and 1% formic acid in water at a flow-rate of 10 μl/min. The orifice voltage was set to 80 V and the ionspray potential to 4500–5000 V.

RESULTS AND DISCUSSION

The rationale for adopting HPDC for mapping the peptides formed by tryptic digestion of a protein is to characterize the minor components of the mixture whose presence conveys information about the composition of the protein preparation digested and also about the specificity of the enzymatic digestion and side-reactions occurring in the digestion. Chief among the objectives of examining the "fine structure" in the enzymatic map is to identify the tell-tale peptides indicating the presence of protein variants in the preparation. These variants can arise from degradative processes acting on the protein, such as deamidation, oxidation and proteolysis, from incorrect folding and disulfide rearrangement, from errors in translation by the host cell [27], from chemical modifications to the protein caused by processing conditions used, for example, to cleave the target protein from a larger fusion construct, or from the heterogeneity conferred on a glycoprotein by the covalently linked carbohydrate structures. Other peptides occurring at low levels in the tryptic map may arise as artifacts of the digestion process, resulting either from autoproteolysis of trypsin [28] or from non-specific cleavages of the protein that are observed even in highly purified preparations of proteolytic enzymes. Finally, host cell proteins may also contribute to the low-level contaminants found in the peptide mixture, although in a pharmaceutical-grade protein these contaminants are expected to be present at undetectable levels [29]. Hence, the information content of the minor components of a mixture can be sufficient to warrant development of methods, such as HPDC, for closer examination of the trace level components of the mixture.

A chromatogram of the tryptic digest mixture of rhGH run under conventional gradient elution conditions is shown in Fig. 3A. Table I shows the identities and masses of the major peptides expected from the map, designated numerically according to the position of the peptide in the linear sequence of the protein [26]. The peptides labelled $T10c_1$ and $T10c_2$ are fragments resulting from a chymotryptic-like clip in the T10 peptide that is one example of the non-specificity of the cleavage observed with trypsin. Fig. 3B is an enlargement of the baseline of Fig. 3A, showing the presence of numerous small peaks that presumably convey certain of the information described above. As the small peaks are present at such low levels, they are not readily characterized from the tryptic map to extract this information.

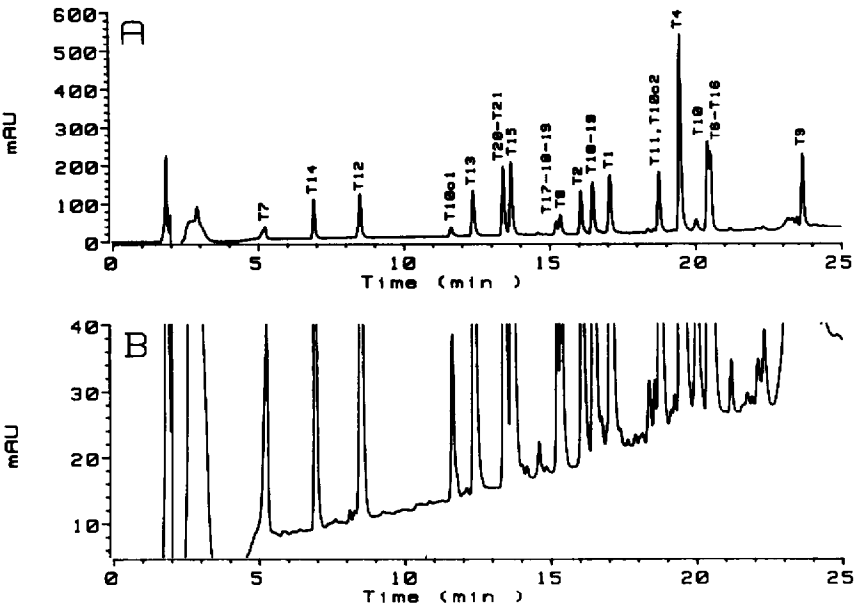

Fig. 3. (A) Absorbance profile of the tryptic map of rhGH obtained by gradient elution chromatography. (B) The same chromatogram at lower attenuation, showing the minor component peaks occuring in the tryptic map.

TABLE I

PEPTIDES PRODUCED BY DIGESTION OF HUMAN GROWTH HORMONE WITH TRYPSIN

$[M+H]^+$ is the calculated molecular weight for the most abundant monoisotopic species.

Identifier	Residues	$[M+H]^+$	Sequence[a]
TI	1–8	930.54	FPTIPLSR
T2	9–16	979.50	LFDNAMLR
T3	17–19	383.21	AHR
T4	20–38	2342.14	LHQLAFDTYQEFEEAYIPK
T5	39–41	404.22	EQK
T6[b]	42–64	2616.24	YSFLQNPQTSLCFSESIPTPSNR
T7	65–70	762.36	EETQQK
T8	71–77	844.49	SNLELLR
T9	78–94	2055.20	ISLLLIQSWLEPVQFLR
T10	95–115	2262.13	SVFANSLVYGASDSNVYDLLK
$TIOc_1$	96–99	537.27	SVFAN
$TIOc_2$	100–115	1743.90	LWGASDSNVYDLLK
TII	116–127	1361.67	DLEEGIQTLMGR
T12	128–134	773.38	LEDGSPR
T13	135–140	693.39	TGQIFK
T14	141–145	626.32	QTYSK
T15	146–158	1489.69	FDTNSHNDDALLK
T16[b]	159–167	1148.55	NYGLLYCFR
T17–T18–TI9	168–178	1381.71	KDMDKVETFLR
T18–T19	171–178	1253.62	DMDKVETFLR
T20[c]	179–183	618.34	IVQCR
T21[c]	184–191	785.31	SVEGSCGF

[a] Single-letter coding for amino acids used.

[b] T6 and T16 are disulfide-linked, with a total $[M+H]^+$ of 3762.8.

[c] T20 and T21 are disulfide-linked, with a total $[M+H]^+$ of 1401.4.

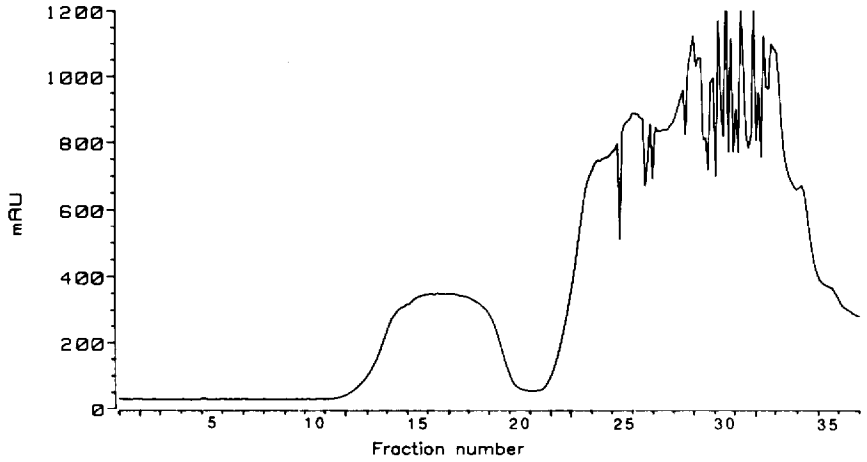

Fig. 4. Displacement chromatogram of 9 mg of rhGH tryptic peptides. The displacer concentration was 3 mg/ml. Fraction collection commenced at 309 min, and 1-min fractions were collected. The chromatogram shown was produced by summing the absorbance profiles obtained at 214, 254 and 280 nm.

In order to develop HPDC tryptic mapping conditions, an essential requirement is to identify a substance suited to displacement of the peptide mixture. The required characteristics of a displacer have been described [9], and several potential displacers of peptides have been reported [1–6]. One of these displacers, CTAB, has been employed as a displacer in this application [18] and so was used here to displace a feed consisting of 9 mg of the tryptic peptide mixture. The resulting displacement train is shown in Fig. 4. The angular shape of the profile suggests that the components of the mixture have formed a classical displacement train, although non-ideal effects at these concentrations disturbed the absorbance detection. The HPLC analyses of fractions collected during the displacement separation are shown in Fig. 5. Roughly

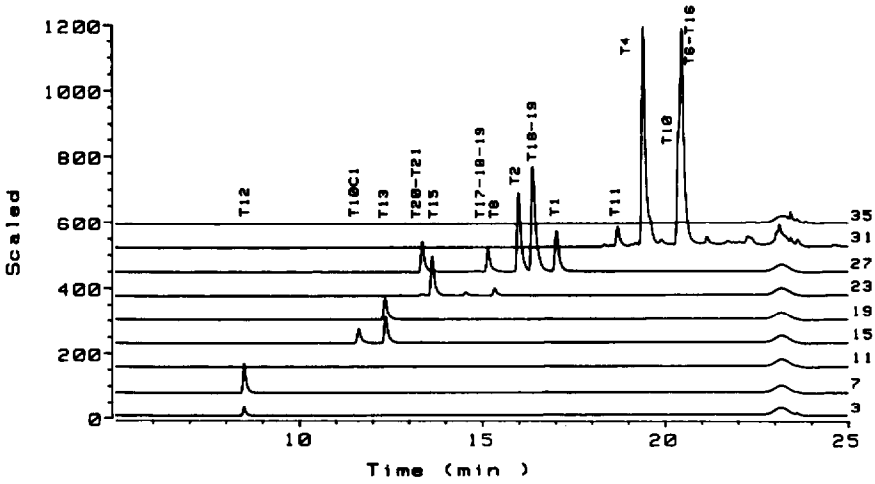

Fig. 5. HPLC of the fractions collected during the displacement separation shown in Fig. 4. The fraction number is indicated adjacent to each chromatogram.

similar trends are observed in the relative retention behavior of peptides in elution chromatography, employed for the analyses of fractions, and in the HPDC separation. Thus, the peptides that elute early in the tryptic map are also those which occur early in the displacement train. There are exceptions to the parallelism of relative retention in the two modes. One example is the peptide identified as T20–T21 in Fig. 5, which elutes ahead of T15 in the linear elution tryptic map, but occurs behind T15 in the displacement train, as indicated by its appearance in fraction 27 and absence from fraction 23. The later fractions in Fig. 5 are seen to contain a considerable number of peptides, indicating that bands in this part of the displacement train are highly concentrated and narrow, relative to the 1-ml volume of the fractions. As expected, the rear of the displacement train contains the most strongly retained peptides and these form the most concentrated bands. This portion of the chromatogram also reveals that essentially all of the peptides through T6–T16 are effectively displaced by CTAB, and the only major peptide absent from the displacement train is the most hydrophobic, T9, which is poorly soluble in aqueous buffers. The absence of peptides from fraction 35 indicates that the displacement train components did not tail into the displacer zone, which lends support to the conclusion that CTAB efficiently displaced the peptide mixture under these conditions.

Mass spectrometry offers an alternative approach for the rapid analysis of the fractions collected in the HPDC analysis and has the additional benefit of providing mass information that can aid in identification of peptides in the mixture. Aliquots of fractions 15 and 23 were analysed by electrospray MS, yielding the spectra shown in Fig. 6 and 7, respectively. In each of the mass spectra the masses corresponding to major constituents of the digest mixture are identified. The chromatogram of fraction 15 shown in Fig. 5 reveals the presence of two main constituents, the peptides T10c$_1$ and T13, which also dominate the spectrum in Fig. 6 by their molecular ions with m/z 537 and 693, respectively. In addition to these molecular ions, the doubly charged T13

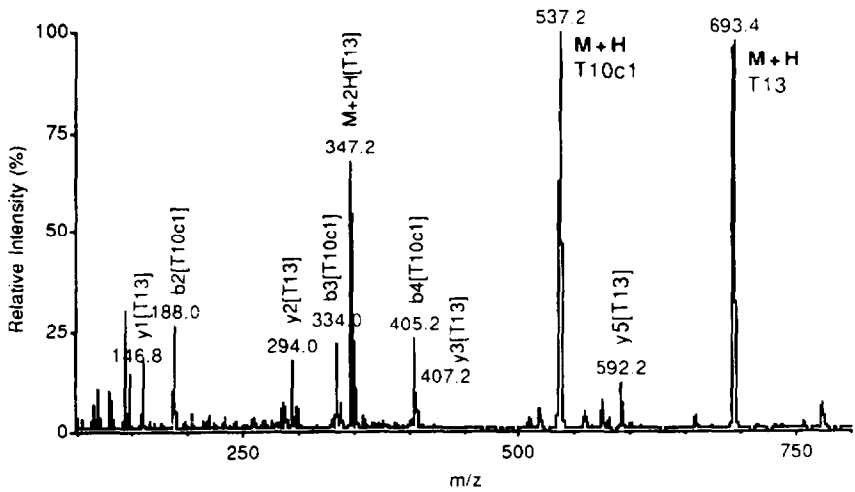

Fig. 6. Mass spectrum of fraction 15. Ions arising from T10c$_1$ and T13, including fragment ions, are identified.

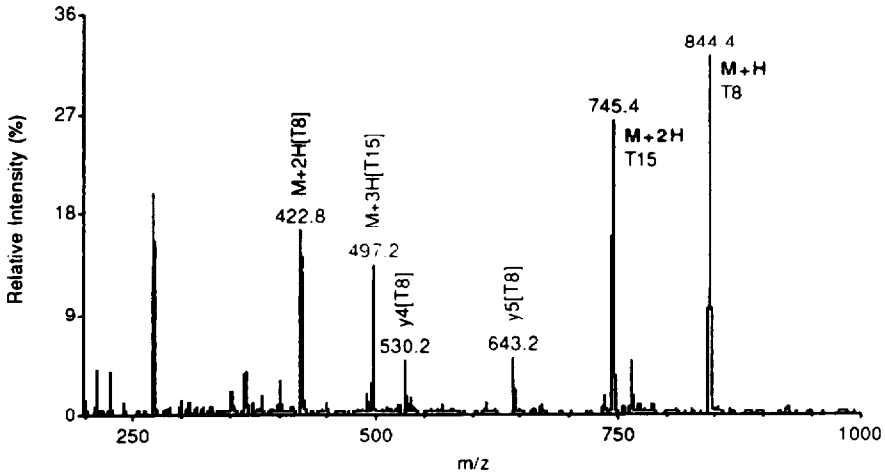

Fig. 7. Mass spectrum of fraction 23. Ions arising from T15 and T8, including fragment ions, are identified.

ion (m/z = 347) is apparent along with several ions that are products of collision-induced dissociation in the electrospray interface [19,20]. Such fragmentation is an artifact of the electrospray ionization mechanism even at the low orifice potential employed in these analyses and can provide additional sequence information to aid in the identification of the molecular ion, as discussed further below. In addition to the ions derived from the two major peptides in this mixture, there are ions present at

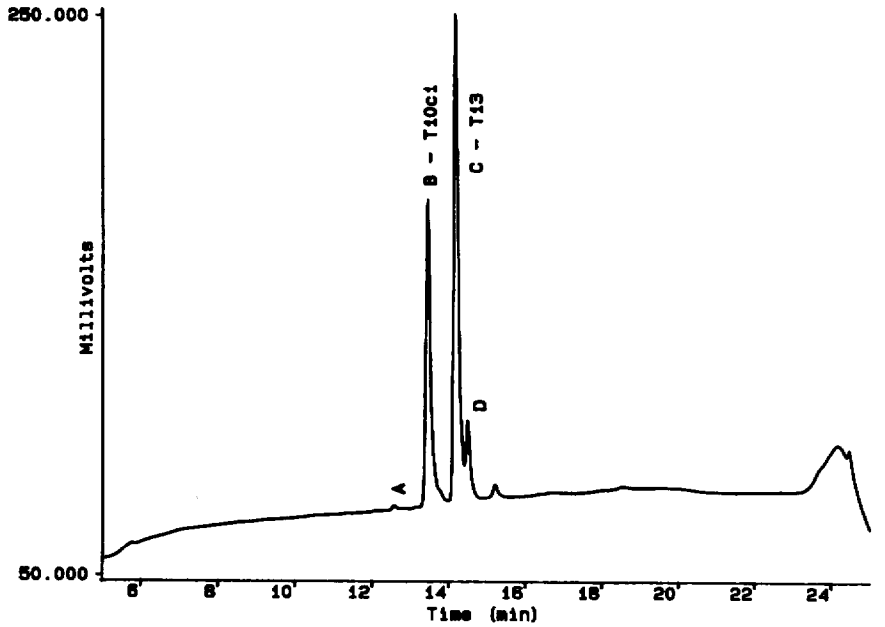

Fig. 8. Reversed-phase HPLC of fraction 15 of the HPDC tryptic map. Peptides collected for identification by mass spectrometry are labelled to correspond with the appropriate spectrum in Fig. 9.

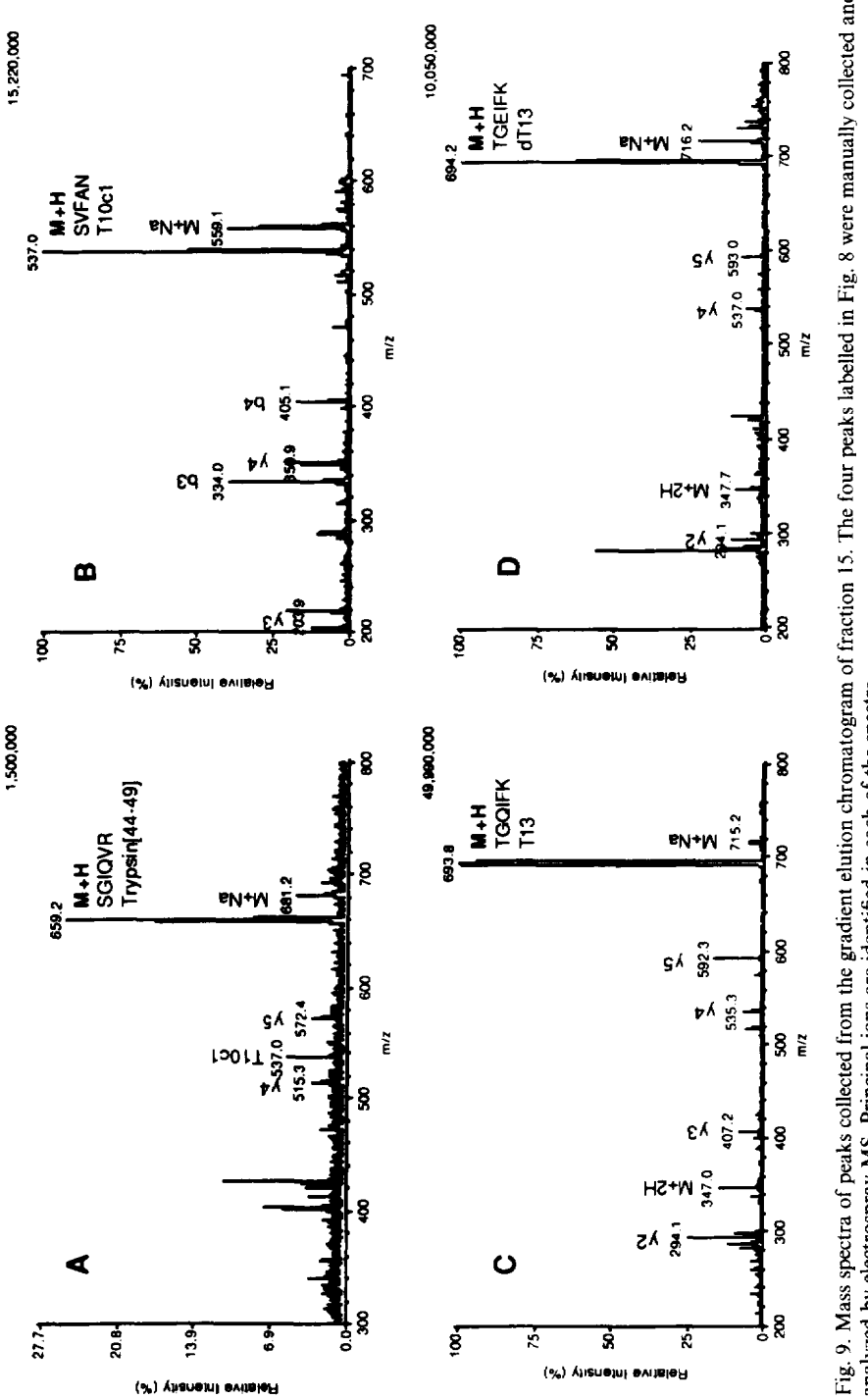

Fig. 9. Mass spectra of peaks collected from the gradient elution chromatogram of fraction 15. The four peaks labelled in Fig. 8 were manually collected and analyzed by electrospray MS. Principal ions are identified in each of the spectra.

much lower abundances that presumably represent components of the fraction present at low concentrations, including peptides present as minor peaks in the chromatogram of the mixture in Fig. 5. The mass spectrum of fraction 23 shown in Fig. 7 yields an analogous picture. The chromatogram of fraction 23 shown in Fig. 5 contains the two tryptic peptides, T15 and T8. The mass spectrum of the mixture is likewise dominated by molecular and fragment ions of these two peptides. Also present, as in Fig. 6, are ions that are not readily associated with peptides expected to arise from typtic mapping of rhGH, and whose identification may warrant further investigation.

As the fractions themselves represent relatively complex mixtures, this investigation is hampered by limitations on the interpretation of mass spectral data such as described above. Therefore, the identification of these minor components requires further purification and isolation of individual species. As argued above, identification of the peptides corresponding to these peaks is expected to yield information about the composition of the protein preparation and about the trypsin digestion process. To illustrate the isolation by HPDC of useful amounts of minor components of the peptide digest, individual fractions from the separations shown in Fig. 5 were rechromatographed by gradient elution HPLC and the resulting peaks were manually collected for subsequent analysis by electrospray mass spectrometry. Figure 8 shows the four peaks collected by gradient elution HPLC analysis of fraction 15 of the displacement separation. The mass spectra of the individual peaks are shown in Fig. 9. Certain of the assignments of peaks in Fig. 8 correspond to peptides already identified from the conventional tryptic map shown in Fig. 3. Thus, T10c$_1$ (m/z = 537) and

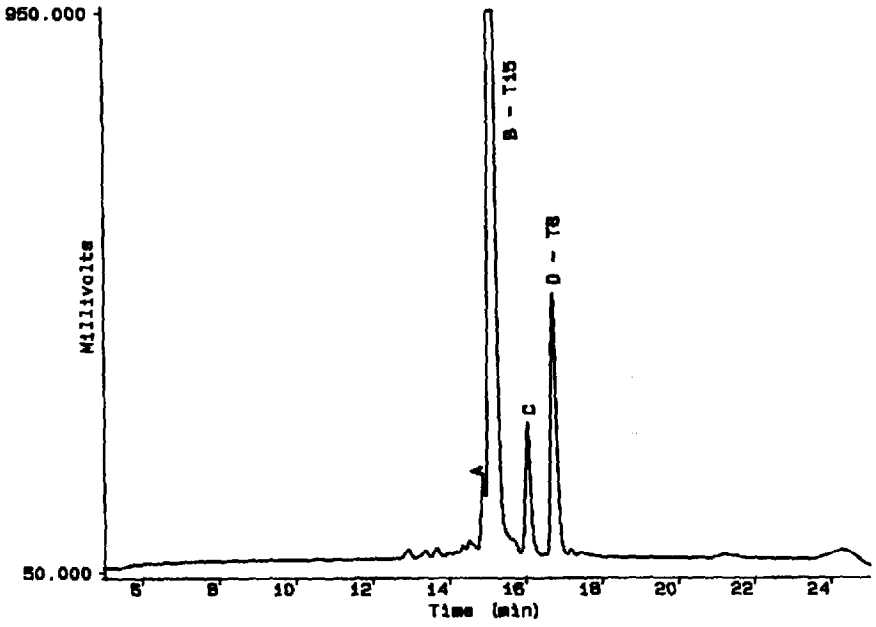

Fig. 10. Reversed-phase HPLC of fraction 23 of the HPDC tryptic map. Peptides collected for identification by mass spectrometry are labelled to correspond with the appropriate spectrum in Fig. 11.

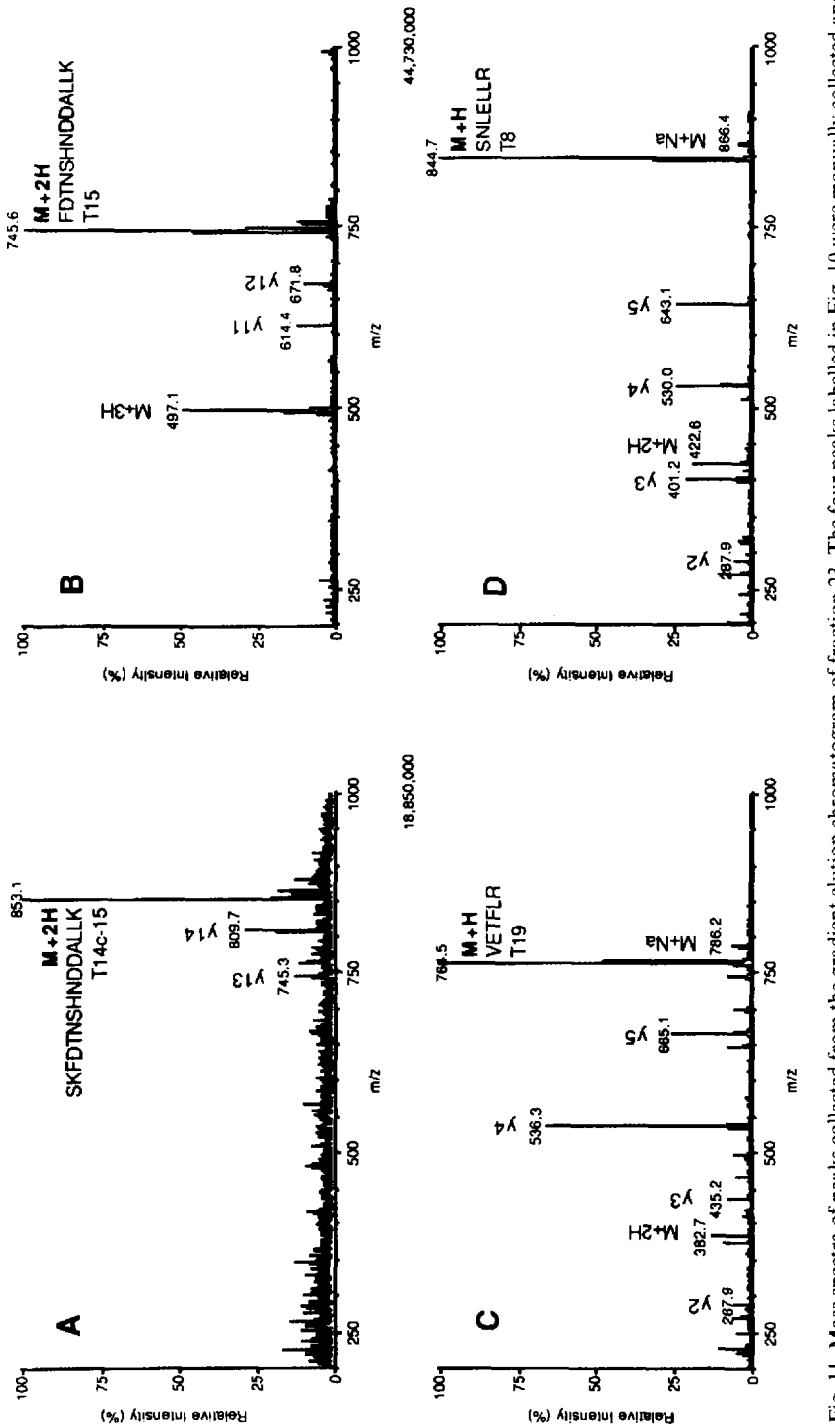

Fig. 11. Mass spectra of peaks collected from the gradient elution chromatogram of fraction 23. The four peaks labelled in Fig. 10 were manually collected and analyzed by electrospray MS. Principal ions are identified in each of the spectra.

T13 (m/z = 693) are readily identified by molecular ion information and confirmed by fragment ion identification.

The assignments of fragment ions for these purified peptides is less tentative than in the spectrum shown in Fig. 6, which is the mass spectrum obtained by analysis of a mixture. The fragment ions observed are related to the parent ion peptide sequence with, for instance, predominantly *y*-series ions, in the standard nomenclature [30], observed in the T13 spectrum. *y*-Series ions are C-terminal fragments, and arise from maintenance of the positive charge on the C-terminal lysine of this tryptic peptide. Conversely, the spectrum of $T10c_1$, which lacks a C-terminal basic residue, contains predominantly *b*-series ions, *i.e.*, N-terminal fragments, which occur owing to maintenance of the positive charge at the N-terminus of the fragmented peptide. The other peptides in fraction 15 are relatively minor components of the tryptic digest mixture. Among these is a deamidated variant of the T13 peptide (m/z = 694). T13 contains a single glutamine residue that can undergo deamidation to a relatively small extent under the alkaline conditions employed for digestion with trypsin. Deamidation converts the glutamine residue to glutamic acid, resulting in a unit increase in the mass of the deamidated peptide that can be resolved with the mass spectrometer. Comparison of the fragment ions arising from the deamidated T13 with those of the native T13 peptide confirms that the unit increase in mass is associated with the glutamine residue in the peptide, as can be seen by comparing Fig. 9C and D. The deamidated peptide is resolved from the native T13 peptide in the reversed-phase chromatogram, but was not resolved in the mass spectrum of the mixture, as seen in Fig. 6. Thus, identification of this important degradation product is best carried out by sequential chromatographic separation and mass spectral analysis, rather than by MS alone.

Also contained in fraction 15 is a peptide arising from autolysis of the trypsin employed for digestion of the protein. The spectrum of the trypsin-derived peptide (m/z = 659) is shown in Fig. 9A and corresponds to residues 44–49 of the sequence of bovine pancreatic typsin [28]. This assignment of the molecular ion is supported by sequence data obtained by assignment of the fragment ions arising from collision-induced dissociation in the electrospray interface [19,20]. Hence, the molecular ion with m/z 659 corresponds to the peptide with sequence SGIQVR, using the single-letter code, in trypsin. Accompanying the molecular ion are fragment ions with m/z 572 (corresponding to the y_5 fragment ion), 515 (y_4) and 402 (y_3). The coincidence of these ions with the molecular ion confirms the identification of this peak as a peptide arising from autolysis of trypsin.

Fig. 10 shows the peaks collected from the chromatogram of fraction 23. The identifications of the major peaks as the doubly charged T15 (m/z = 745) and the singly charged T8 (m/z 844) were confirmed by their mass spectra, as shown in Fig. 11B and D. Two minor peaks were also identified, and corresponded to cleavages that are not commonly seen in trypsin digestion of rhGH. The earlier eluting of these peaks, with m/z 853, was identified by MS as a doubly charged peptide constituting residues 144–158 of hGH, and represents a chymotryptic-like clip between Tyr-143 and Ser-144 in what would be the T14 peptide. Residue 145 is a lysine, but no cleavage occurred after this residue, suggesting that this amide bond, occurring near the clipped portion of T14, is a poor substrate for trypsin. The fragmentation pattern discerned for this peptide and shown in Fig. 11A confirms the identification of this

relatively unusual product of tryptic digestion. Analysis of all the fractions collected idicates that this peptide constitutes *ca.* 0.9% of the T15 residues in the digest mixture, demonstrating the high resolving power of HPDC for minor components of a complex misture. The other minor peptide identified in fraction 23 is T19, which is usually not cleaved from T18 by trypsin, as indicated in Fig. 3 and Table I. The fragmentation pattern observed in Fig. 11D confirms that this peptide is present in the digest of hGH. Integration of the chromatograms of fractions containing this peptide shows that it occurs at a level of *ca.* 1.5% of the T19 residues in the protein, compared with *ca.* 26.5% present in the T18-19 peptide and the balance in the T17–18–19 peptide.

CONCLUSIONS

HPDC permits the recovery of useful amounts of minor components of the peptide mixture produced by digestion of a protein with trypsin. Characterization of these trace component peptides can provide information on the enzymatic properties of trypsin and on the detailed nature of the protein substrate. HPDC tryptic mapping can be employed to identify variants constituting less than 1% of the protein preparation. Hence, tryptic mapping by HPDC has a comparable sensitivity to successful analytical methods involving chromatography of the intact protein, and significantly improved sensitivity compared with conventional gradient elution tryptic mapping. Mass spectrometry is a rapid and efficient means of identifying the minor peptides isolated by HPDC tryptic mapping. Molecular ion fragmentation patterns occurring during electrospray ionization MS provide compelling additional support for the identification of peptides in a mass spectrum.

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