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Investigation of the electrospray response of lysine-, arginine-, and homoarginine-terminal peptide mixtures by liquid chromatography/mass spectrometry

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Guanidination has been used to investigate how modification of the lysine ε-amino group into the corresponding guanidino group affects response in electrospray (ES) mass spectrometry (MS). Selected ion monitoring (SIM) analysis of equimolar mixtures containing arginine-, lysine- and the corresponding homoarginine-terminal peptides following liquid chromatography (LC) showed differences in ES response. The ionisation behaviour of the standard peptides is in accordance with the postulated higher stability of the guanidino group present on arginine- and homoarginine-terminal peptides. Modification of the separation conditions employed during LC demonstrates that relative abundances of electrosprayed peptides ions rely mostly on peptide structure. Copyright © 2002 John Wiley & Sons, Ltd.

Since its introduction, electrospray mass spectrometry (ES-MS) has drastically changed the face of modern mass spectrometry.[1] The ability to transfer highly polar and non-volatile compounds directly into the gas phase as ions enables direct analysis of large molecules dissolved in solution. The ES process is initiated with the production of charged droplets in proximity of the electrospray metal capillary tip held at high voltage. Subsequent shrinkage of the charged droplets is the intermediate step leading to formation of gas-phase ions. Two principal mechanisms have been proposed to explain the ion formation at microscopic level. In the first model, also known as the charge residue model (CRM), the concomitant effects of solvent evaporation and repulsion due to the charge density continue until the remaining particle contains just one ion.[2] In the ion evaporation model (IEM), the increase in surface charge density is regarded as the result of a solvent evaporation. Such a phenomenon triggers a coulombic repulsion that surpasses the adhesion of the particles in the droplet, generating ions, which are singularly ejected from the surface.[3]

Although the two models were proposed more than 20 years ago, they are still under debate, and the lack of general agreement about ion formation has not discouraged practitioners from routinely using ES. Nevertheless, the unpredictable effect of solvent, analyte, and electrolyte on the ES response hampers relative quantification of all components observed in the spectrum.[4] It was previously reported that two analytes, identical in charge and similar in type, at the same concentration could display significantly different ES response.[5] In the case of proteins, the gaussian distribution of the ion signals in ES mass spectra may be dictated by the abundances of preformed, multiply charged species in aqueous solution.[6] When the ion abundance profile reflects the concentration of ionic species in solution, each basic amino acid residue plays an active role in the final charge state distribution. However, for several proteins, significant deviations were detected in their corresponding spectra.[7] In some molecules, not all basic sites are protonated. In others, additional residues must be ionised. Since differences between the theoretical and observed multiply charged envelope were observed, similarities between the ion abundance profile in the mass spectrum and the distribution of preformed multiply charged ions in the aqueous solution seem be more apparent than real. In fact, results from Williams's laboratory[8] indicate that a contribution due to gas-phase reactions between ions and solvent molecules has to be taken into account to determine the maximum charge state of a protein in an electrospray spectrum.

Due to the limited number of amino acid residues, peptides represent a suitable model to investigate further relationships existing between chemical nature of the analyte and its ES response. The solvophobicity of peptide ions has also been shown to be important during ES analysis.[9] Similar to the observations reported for fast atom bombardment (FAB), where spectra are dominated by the ion signals corresponding to the more hydrophobic peptides[10] (according to their Bull and Breese index[11]), the non-polar character of small peptides governs their ES-MS response. Ceck and Enke[9] reported that the variation in non-polar character of six tripeptides has an effect on their ES response and increased detection is observed for peptides with increas-

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ingly non-polar side chains. These findings were rationalised on the basis of the equilibrium-partitioning model, according to which the relationship between composition of the solution and relative abundances of the ions in the mass spectrum depends on the partitioning of the ions between two parts of the droplet.[12] The increased non-polar character of an ion, leading to its enhanced affinity for the surface phase, results in more successful competition for excess charged and higher ES response. Correlation between retention time of peptides in reversed-phase high-performance liquid chromatography (RP-HPLC) and their ES response seem to confirm the preferential ionisation of the more hydrophobic peptides displayed when insufficient extra charges are available.[13]

With the advent of proteomics (study of the full complement of proteins expressed in a cell under a given set of physiological or developmental conditions), electrospray analysis has become a valuable tool for peptide/protein characterisation. In the typical approach, arginine- and lysine-terminal peptides are the hydrolysis products resulting from trypsin-mediated digestion of proteins. Although this class of peptides has been widely analysed by ES-MS, the correlation existing between their structures and the ES response has not been examined systematically. In this paper, guanidination of lysine[14–17] permits evaluation of how conversion of the amino group into a guanidino moiety results in an increased ES response for the corresponding homoarginine-terminal peptide ion.

MATERIALS AND METHODS

O-Methylisourea was obtained from Lancaster (Morecambe, UK). Interleukin (VQGEESNDK), glacial acetic acid and heptafluorobutyric acid (HFBA) were purchased from Sigma (Dorset, UK). The synthetic peptides of sequence AFLDASR and AFLDASK (the peptides are indicated by using one-letter abbreviation for each amino acid residue) were obtained from New England Peptide Inc. (New England, USA). Their purity (>96%) was certified by HPLC and the peptides were used at the concentration of 2–20 pmol/uL without further purification. All experiments were performed on an LCMS-2010 single quadrupole mass spectrometer (Shimadzu, Japan) fitted with an electrospray source. Source conditions were maintained constant during the analysis. The nebulising gas (nitrogen) was used at a flow rate of 4.5 L/min. The probe voltage was set to 4.5 kV, and the source block and source heated desolvation line were operated at 200 °C. Data was acquired in SIM mode for a total of 12 selected ions. The quadrupole array (Q-array) and octapole worked in fix mode using microscans of 2 u. Peptide solutions (5 uL) were loaded using a SIL-10A_{VP} autosampler (Shimadzu). Components were separated on a Phenomenex Luna C_{18} column (2.0 mm i.d. × 50 mm). In gradient mode, mobile phase composition was 0.05% (v/v) trifluoroacetic acid (TFA) for the aqueous phase (A) and 0.05% (v/v) TFA and acetonitrile (9:1, v/v) for the organic phase (B). LC-10AD_{VP} HPLC pumps (Shimadzu) were used to deliver solvent at a flow rate of 250 uL/min. The composition of B was increased linearly for 9 min from 0% to 50%. Gradient profile was controlled by LCMSSolution 2.0 software. All data were processed using PostRun software. For the isocratic separation, mobile phase compositions were derived from the gradient curve considering the retention times at which the peptides of interest were eluted. Guanidination of lysine was performed as described previously,[14] by carefully adding discrete aliquots of NaOH in order to reach the optimum pH.

RESULTS

In matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS), guanidination is beneficial to increased detection of lysine-terminal peptides in tryptic digest mixtures.[14] Guanidination relies on the selective conversion of the lysine side chain amino group into a guanidino moiety (identical to the functional group on the arginine side chain). Although no signals from underivatised lysine-containing peptides are observed during MALDI analysis in the guanidinated mixtures, the variability in MALDI response displayed by pairs of peptides differing only in the presence of homoarginine or lysine residues suggests that unreacted lysine-terminal peptide may still be present and undetectable due to the poorer ionisation efficiency of the lysine-containing peptide ions.

Previous studies reported that guanidination of tryptic digests results in complete conversion of the lysine-terminal peptides under ES analysis.[18] In order to verify the absence of starting material, lysine-containing peptides treated with *O*-methylisourea were loaded onto a C_{18} column and subjected to liquid chromatography prior to ES analysis. The separation has two advantages: first, it removes the excess of *O*-methylisourea, which interferes with the ionisation process. Secondly, all components in the reaction mixture can be resolved and eluted separately into the mass spectrometer. Subsequent detection by ES-MS allows comparison and quantification of the relative amounts of lysine- and its homoarginine-terminal counterpart. Figure 1 illustrates LC/ES-MS analysis of lysine-terminal standard peptides treated with methylisourea. Selected ion monitoring (SIM) of four specific marker ions corresponding to the singly and doubly charged ions of lysine- and homoarginine-terminal peptides was used to monitor whether peaks in the total ion chromatogram contain lysine-terminal peptides. Mass spectrometric analysis in full scan mode showed that *O*-methylisourea reacts solely with the lysine amino group; in accordance with previous work,[18] no derivatisation of the N-terminal amino group was found. Figure 1(a) displays the mass chromatogram obtained following LC/MS analysis of 35 pmol of interleukin after guanidination with *O*-methylisourea: two distinctive signals corresponding to the singly and doubly charged ions of the homoarginine-terminal peptide (*m/z* 1047.40 and 524.20, respectively) are observed. Similarly, the ion chromatogram monitoring the guanidination of AFLDASK shows no traces of the unreacted lysine-terminal peptide (expected at *m/z* 751.25 and 376.2, respectively) and only the two ion signals belonging to the homoarginine-containing counterparts are detected (*m/z* 793.35 and 397.25). In Fig. 1 the arrows indicate the expected retention times of the unreacted lysine-terminal peptides obtained from the LC/MS analysis under identical condi-

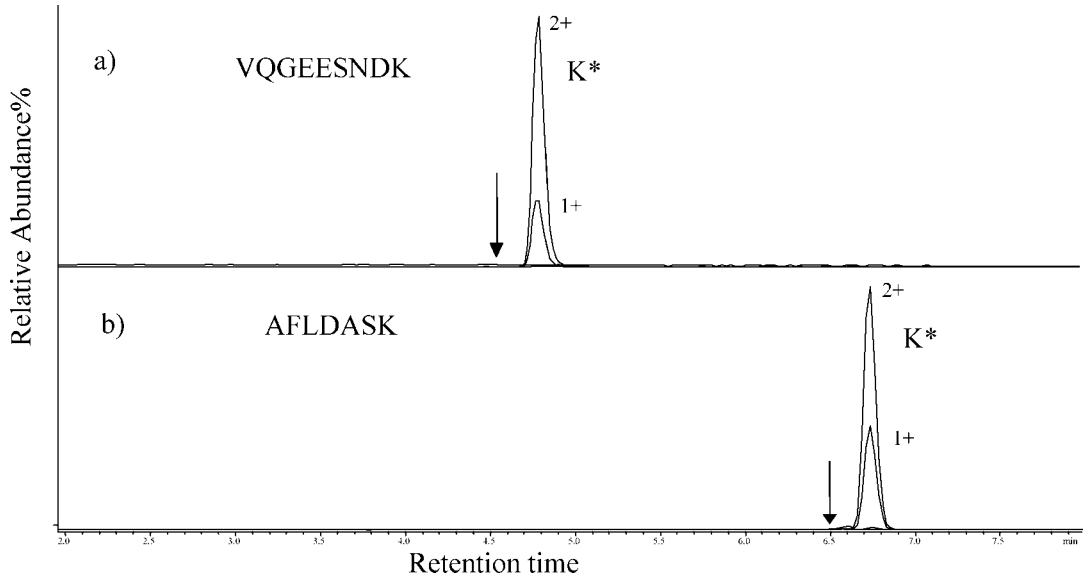

Figure 1. LC/ES-MS analysis of lysine-terminal standard peptides treated with *O*-methylisourea. Selected ion monitoring (SIM) of four marker ions is performed selecting the singly and doubly charged ions of lysine (K) and its corresponding homoarginine-terminal peptide (K*). (a) 35 pmol of interleukin, $[M + 2H]^{2+}$ and $[M + H]^{+}$ at *m/z* 503.2 and 1005.4 are indicated by 2+ and 1+, respectively. (b) 30 pmol of AFLDASK $[M + 2H]^{2+}$ and $[M + H]^{+}$ at *m/z* 376.25 and 751.25 are indicated with 2+ and 1+, respectively. The arrows indicate the expected retention times of the unmodified lysine-containing peptides.

tions. The results indicate that the high reactivity of lysine amino groups towards *O*-methylisourea produces peptide mixtures containing exclusively homoarginine-terminal homologues. Although different organic solvents were tested to improve chromatographic separation, modification of mobile phase composition has shown no effect on the relative abundances of singly and doubly charged peptide ions. For both peptides, doubly charged ions remain the most abundant species in the mass spectrum.

Binary equimolar mixtures containing lysine-terminal peptides and the analogous homoarginine-terminal counterparts were prepared and comparisons between the two ion

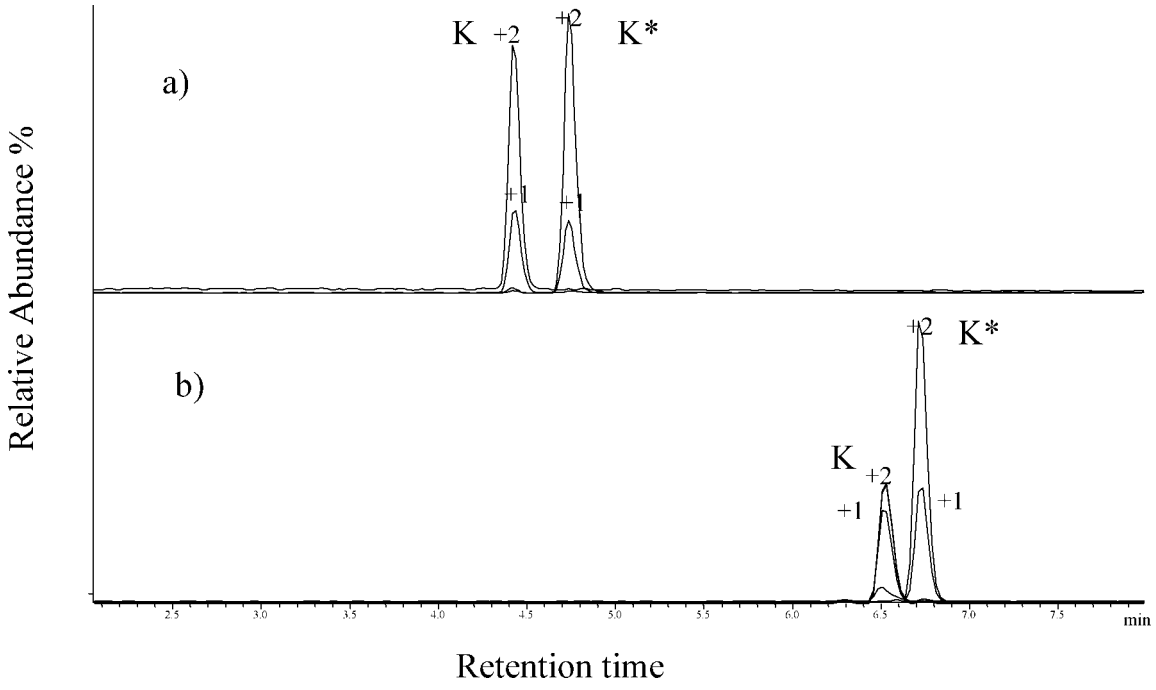

Figure 2. (a) LC/ES-MS analysis of an equimolar mixture of interleukin (K, 12.5 pmol) with its guanidinated counterpart (K*, 12.5pmol) acquired in selected ion monitoring. Doubly charged ion signals $[M + 2H]^{2+}$ are indicated by +2 while singly charged ion signals $[M + H]^{+}$ are indicated by +1. (b) SIM LC/ES-MS analysis of a equimolar mixture of 20 pmol of AFLDASK and AFLDASK*.

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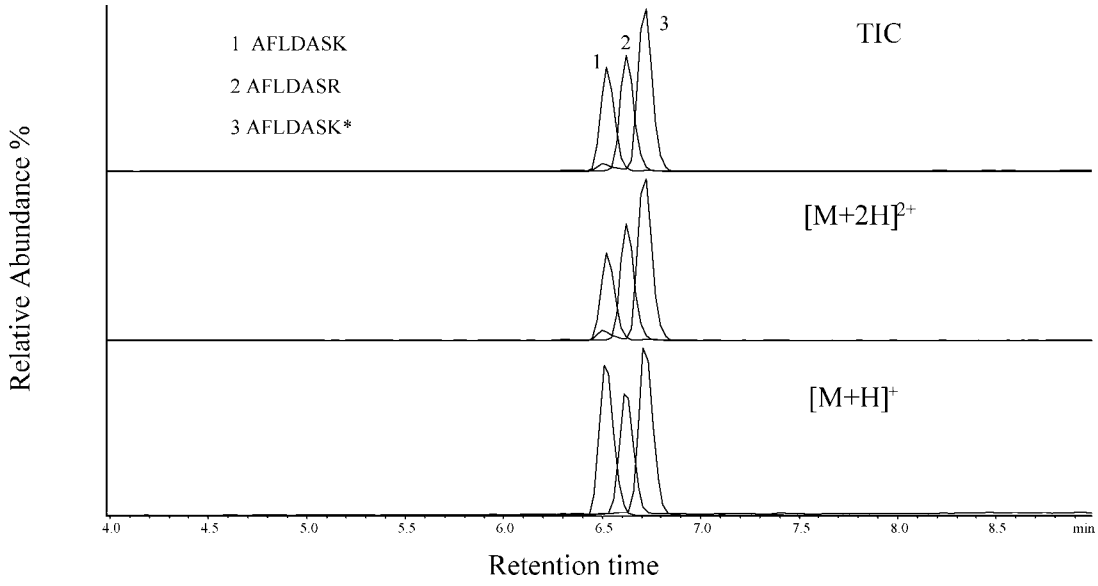

Figure 3. LC/ES-MS analysis of a solution containing equal amounts (16.6 pmol) of AFLDASK, AFLDASR and the homoarginine-terminal analogue, AFLDASK*. The lysine-terminal peptide is eluted first followed by the arginine-terminal counterpart. SIM analysis of all six ions indicates that the homoarginine-terminal peptide ions are the dominant signals in the ion chromatogram, regardless of the charge state selected.

signals were performed. Figure 2(a) shows the mass chromatogram of 12.5 pmol each of interleukin and its guanidinated analogue during LC separation followed by ES-MS. The conditions employed during the chromatographic separation (composition of mobile phases, gradient profile, etc.) were kept identical to those used in the previous experiment (see Fig. 1). Total ion chromatogram (TIC) traces of homoarginine-terminal peptides (corresponding to the sum of the singly and doubly charged ion signals) have higher relative abundance than lysine-terminal ones. The main contribution is due to the relative ES response of the doubly charged ion peaks. This observation is also made with an equimolar mixture of AFLDASK and its guanidinated counterpart AFLDASK* (Fig. 2(b)). The two peptides (AFLDASK and AFLDASK*) display larger difference in response between the doubly protonated ion populations. The difference in relative intensities between the two singly charged ion signals is not as marked as for the doubly charged species. Discrepancy in ionisation behaviour between the interleukin-related peptides and AFLDASK/AFLDASK* reflects the substantial contribution made towards electrospray response by peptide structure. Although the gas-phase basicity of the C-terminal amino acid plays the main role in influencing the ES response, other parameters, including greater abundance of highly polar amino acid residues and the effect of such amino acids on the peptide conformation, must be taken into account in defining the overall ionisation efficiency of the species under analysis.

To investigate how minor variations in peptide structure

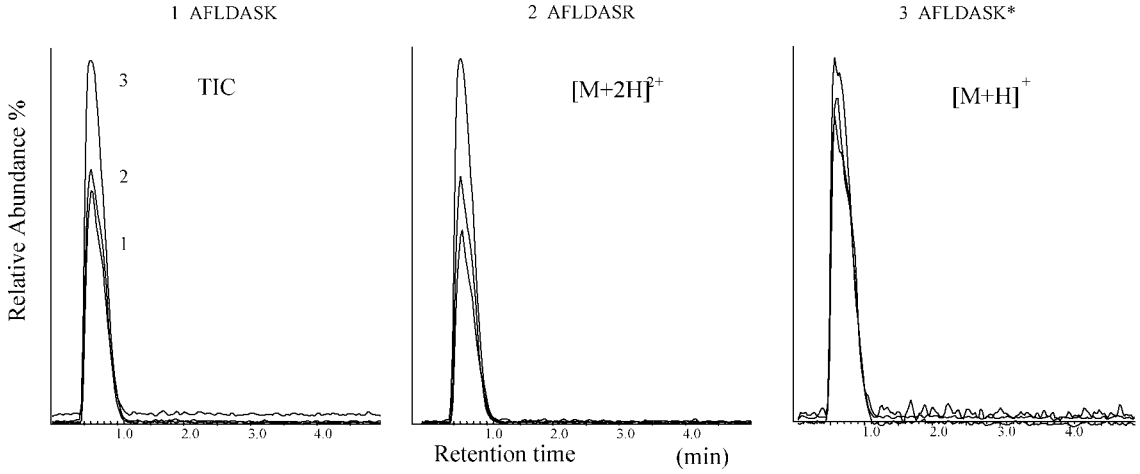

Figure 4. Isocratic separation of a solution containing equal amounts (16.6 pmol) of AFLDASK, AFLDASR and the homoarginine-terminal analogue, AFLDASK*. The conditions employed correspond to the percentage of mobile phase B (36%) at which the peptides were eluted in gradient mode.

may affect signal intensity, the arginine-terminal analogue AFLDASR was added to the mixture. Figure 3 indicates the ion chromatograms of a solution containing 16.6 pmol each of AFLDASK, AFLDASR and the homoarginine-terminal derivative AFLDASK* separated by LC prior to MS detection. The arginine-terminal peptide displays an ion chromatogram with abundance higher than the corresponding AFLDASK in accordance with the higher basicity of the arginine. By comparing the two sets of peptide ions produced by AFLDASR and AFLDASK*, the latter generally displays ion signals with higher intensity. Unlike the lysine- and arginine/homoarginine-terminal peptides (AFLDASK vs AFLDASR/AFLDASK*), where the difference in ionisation efficiency can be related to the higher stabilisation of the protonated peptide (due to the higher basicity of arginine/homoarginine side chain), in the case of the AFLDASR and AFLDASK* the difference in ES response cannot be simply related to the effect of the guanidino group as both are identical. The difference may be attributed to the increased level of proton solvation provided by the extra methylene group in the peptide backbone. This result can be correlated with what was previously reported on the fragmentation efficiency of these peptides.[19] Using energy-resolved mass spectrometry (ERMS), fragmentation efficiency curves for AFLDASR/AFLDASK/AFLDASK* revealed an unexpected shift in collision energy for the homoarginine-terminal peptide suggesting that intramolecular interactions provides higher stabilisation of the peptide ion. Mobile phases containing other types of acids were used to separate the mixtures. In the aqueous and organic phase, TFA was replaced with 0.005% (v/v) HFBA + 0.4% (v/v) acetic acid. Under these conditions, LC/MS analysis of equimolar mixtures of AFLDASR/AFLDASK produced ion chromatograms in which the arginine-terminal peptide ion is still detected with higher abundance than the lysine-terminal counterpart (data not shown).

Quantification of the difference in ES response following LC allows assessment of whether simultaneous ionisation of the three peptides AFLDASK/AFLDASR/AFLDASK* is detrimental to the relative abundance of the ion peaks monitored. The effect of co-elution of three peptides on their ES response was studied further. The mixture previously analysed (see Fig. 3) was run isocratically. The mobile phase was prepared with approximately the same composition used to elute AFLDASR, AFLDASK and AFLDASK* in the gradient mode (36% B). Hence, no separation is achieved and all three peptides elute simultaneously into the mass spectrometer. Figure 4 displays mass chromatograms of AFLDASK, AFLDASR and AFLDASK* for singly and doubly charged protonated ions. None of the ion signals in the mass chromatogram are suppressed compared with the ion signals observed in the LC/MS experiment (see Fig. 3). The co-presence of three peptides differing in gas-phase basicity shows no effect on their ES response and no apparent signal suppression is detected. This simple experiment demonstrates that response in electrospray relies mostly on the peptide structure and is not significantly affected by co-ionising peptides when they are present in equal amounts.

CONCLUSIONS

Peptide ions bearing the guanidino group are more abundant in the spectrum than the analogue lysine counterparts. This is believed to be attributable to the higher stabilisation of the protonated guanidino moiety, but other factors must be considered. Relative intensities of ion signals corresponding to arginine and lysine analogues are not affected by the different acid conditions employed. Unlike interleukin, in which guanidination of lysine is not accompanied by a considerable improvement in ion abundance for the resulting homoarginine-terminal peptide ion, the comparison between AFLDASR and AFLDASK* indicates that the presence of an extra methylene group significantly alters ES response. Furthermore, application of different conditions to the chromatographic separation step suggests that the ionisation efficiency of arginine-, lysine-, and homoarginine-terminal peptides is dictated by their structure more than by the co-presence of other components.

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