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REGIONAL DISTRIBUTION OF THE Mr 15,000 SOMATOSTATIN PRECURSOR, SOMATOSTATIN-28 AND SOMATOSTATIN-14 IN THE RAT BRAIN SUGGESTS A DIFFERENTIAL INTRACELLULAR PROCESSING OF THE HIGH MOLECULAR WEIGHT SPECIES

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SUMMARY : Three different forms of immunoreactive somatostatin (Mr 15,000, 3,000 and 1,600) were immunologically detected in extracts made from six neural structures of the rat brain. The largest represents the proform, while the smaller were identified by high pressure liquid chromatography with bovine somatostatin-28 (S-28) and -14 (S-14) respectively. In each of the brain structures studied highly variable proportions of precursor, S-28 and S-14 were found. These observations provide suggestive evidence that the intracellular processing of the 15,000 Mr proform may occur differently in the various somatostatinergic pathways of the brain. They argue in favor of a biological role for the precursor in providing distinct relative proportions of S-14 and S-28 in specific rat brain regions.

Somatostatin, originally identified in the hypothalamus (1), is a tetradecapeptide exhibiting numerous biological functions (2) and which is widely distributed throughout the gut, the pancreas, and both the central and peripheral nervous systems (for a review, see 3). The tetradecapeptide, somatostatin-14 (S-14), seems to derive from a proform (Mr 15,000) detected and characterized in the hypothalamus (4, 5). The primary structure of proforms from the pancreas have been deduced from the established sequences of cloned anglerfish or human cDNAs (6,7). In these two species, the large N-terminal precursor domain appears to be conserved. This suggests that this region of the molecule may retain some unknown biological role. Another form, somatostatin-28 (S-28), was isolated from porcine intestine (8) then from ovine hypothalamus (9). Its aminoacid sequence appears to correspond to the C-terminal end of the prosomatostatin precursor as inferred from a comparison with the predicted sequence of pancreatic prosomatostatin. Moreover, it has been reported to exhibit a greater potency than S-14 both in several bioassays and receptor binding experiments (10, 11). Since both S-14 and S-28 may accomplish distinct biological functions

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and are readily detectable by radioimmunoassay, we studied the distribution of the different forms of immunoreactive somatostatin in various areas of the rat brain where somatostatinergic innervation is either intrinseque or extrinseque (3). The results will be discussed in relation with a possible biological role of each of these different somatostatin-containing species in the brain areas which have been studied.

MATERIAL AND METHODS :

Phenylmethylsulfonylfluoride (PMSF) and trasylol, were purchased from Sigma (St Louis, MO). Acetronitrile HPLC grade, was obtained from Prolabo. The synthetic somatostatin was furnished by Clin Midy (Montpellier). The antiserum 36-38 was provided by URIA (Dr Dray, Institut Pasteur, Paris). The antiserum Barbara was from Dr C. Kordon laboratory. The analog Tyr-O somatostatin was from Bachem (California).

BRAIN EXTRACTS : Sprague Dawley male rats (100 g) were sacrified by decapitation and the following tissues were immediately dissected : hypothalamus, cerebral cortex, brain stem, cerebellum, amygdala and neurohypophysis. Freshly obtained tissues were immediatly frozen in liquid nitrogen then homogenized with a Potter Elvejhem in 8M urea, HCl 0.1N containing 5 mM PMSF, 130 U/ml trasysol. The extracts were then centrifuged 1 hour at 100,000 g in a SW 50 rotor (Beckman L50) and the supernatant was filtered on a Sephadex G-50 for analysis of immunoreactivity. The recovery of ^{125}I-Tyr-O-somatostatin, added during the extraction, was about 95%.

GEL CHROMATOGRAPHY : Gel filtrations were performed at 2°C on a Sephadex G-50 column (1.2 x 100 cm) in 0.1N HCOOH. The somatostatin immunoreactivity in each fraction was measured by RIA.

The fractions corresponding to the peak Mr 15,000 were pooled, lyophilized, and refiltered on a Sephadex G-75 column (1.2 x 75 cm) equilibrated with 4M urea, HCOOH 0.1N. The non specific effect of urea present in some samples was eliminated by dilution (1:10) in the radioimmunoassay and by testing the influence of urea concentration on the binding of the antigen to the antibody.

SOMATOSTATIN RADIOIMMUNOASSAY (RIA) : RIA of immunoreactive somatostatin forms,and the corresponding controls,were performed as described in (5) using an antisomatostatin antibody (36-38), ^{125}I-Tyr-O-S-14, and synthetic standard S-14. The antibody, directed toward the C-terminal portion of the molecule, recognizes both S-14 and higher molecular weight forms as well. Synthetic S-28, when incubated in the assay, produced a displacement curve which is parallel to that of S-14 showed 100% cross-reactivity (on a molar basis) with S-14. The antibody Barbara (12) directed toward the central portion of the S-14 was also used for the displacement curves of S-14 and the different somatostatin immunoreactive-like material.

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC) : Identification of S-28 and S-14 was carried out by HPLC (SP 8000 spectra physics apparatus)on a reverse phase Lichrosorb 10 RP 18 or 10 RP 8 column (4.6 x 250 mm) from Merck. The column was eluted either isocratically (for S-14) with a mixture containing 35% v/v acetronitrile, 65% amonium acetate 0.05M adjusted to pH 4, or with a linear gradient of 1-propanol (0-40%) for S-28.

RESULTS :

Sephadex G-50 gel chromatography of hypothalamus, cerebral cortex, brain stem, cerebellum, amygdala, and neurohypophysis extracts, revealed three peaks of somatostatin immunoreactivity (Fig. 1). The predominant one coeluted with synthetic S-14 (Mr 1,600), the median peak coeluted with synthetic hypothalamic S-28 (Mr 3,000) while the one near the void volume eluted in a region corresponding to an estimated molecular weight of 15,000. This last finding was observed either by filtration on Sephadex G-50 or G-75 in the absence or presence of 4M urea (not shown). When the Mr 1,600 and 3,000 peaks were eluted on HPLC they were found to exhibit the same retention time as ovine S-14 and S-28 standards respectively (Fig. 2).

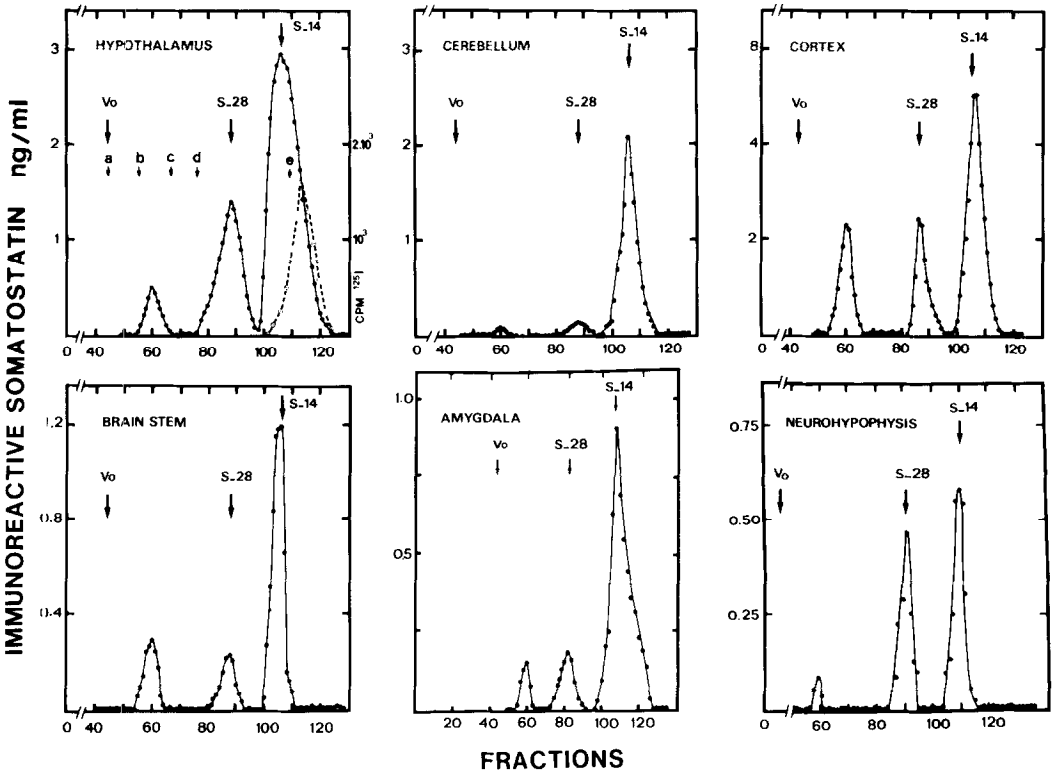

Figure 1 : Elution patterns of immunoreactive somatostatin after filtration of extracts made from various brain regions.

Extracts of a) hypothalamus, b) cortex, c) brain stem, d) cerebellum, e) amygdala, f) neurohypophysis. 1.5 ml of 8M urea extracts (in 0.1N HCl, 0.5 mM PMSF, 130 U/ml trasylol) were fractionated at a flow rate of 5 ml/h on a Sephadex G-50 column (1.2x100cm) equilibrated with 0.1N HCOOH. Somatostatin immunoreactivity (●●●) was measured on an aliquot of 30 µl on each fraction (1 ml) using the antisomatostatin antibody 36-38. Results are expressed in ng/ml/structure except for neurohypophysis for which data are expressed in ng/ml/10 glands. 10^4 cpm of (^{125}I)Tyr-O-S-14 were added as internal marker during the extraction (⊙⊙⊙). Arrows indicate the elution volumes of (a), RNA 16S (Mr ≈ 200,000), (b), HGH (Mr = 22,000), (c), neurophysin II (Mr = 10,000), (d), pancreatic trypsin inhibitor (Mr = 6,000), S-28 (Mr = 3,000), S-14 (Mr = 1,600) and, (e), urea (Mr = 60).

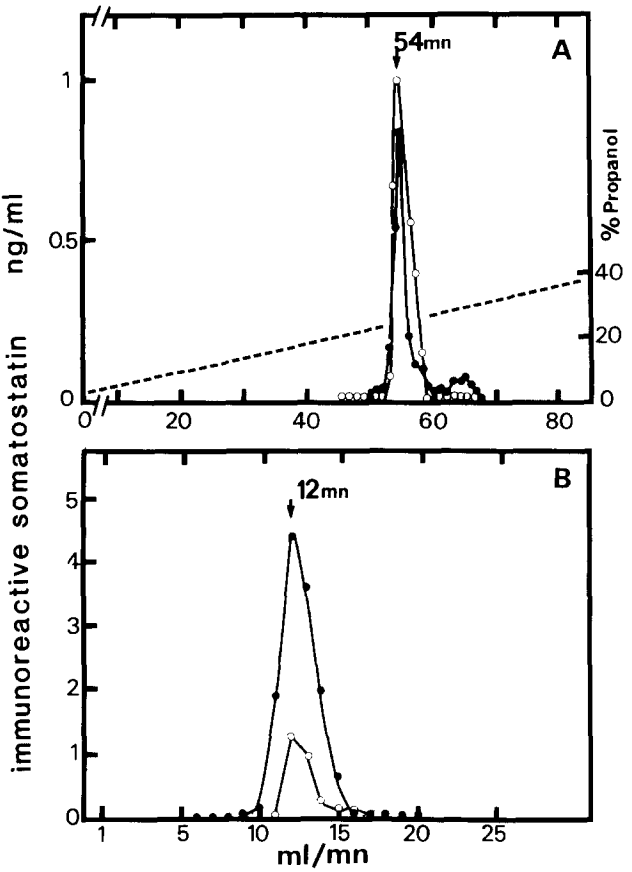

Figure 2 : *High pressure liquid chromatography of S-28 and S-14.*

A) Fractions 88 and 89 recovered from the G-50 column (Fig. 1a) were lyophilized, resuspended in 200 µl of 50 mM amonium acetate pH 4, and analyzed by HPLC on a reverse phase 10 RP 8 column. The sample was eluted by a linear gradient of 1-propanol (0-40%) at a flow rate of 1 ml/mn. Each fraction was assayed by the antisomatostatin antibody 36-38 ; (●●●) endogenous hypothalamic S-28, (O O O) synthetic ovine S-28.

B) An aliquot (750 µl) of fraction 110 recovered from the G-50 column (Fig. 1a) was lyophilized, resuspended in 200 µl of a mixture containing 50 mM amonium acetate, 30% acetonitrile pH 4, and analyzed by HPLC on a 10 RP 18 column eluted isocratically with the same buffer (1 ml/mn). Each fraction was assayed by the antisomatostatin antibody 36-38 ; (O OO) endogenous S-14, (●●●) synthetic ovine S-14.

The profiles of immunoreactivity observed on gel filtration and the relative amounts of the three somatostatin species (Table 1) were found highly variable from tissue to tissue. Although the Mr 1,600 form was the predominant species in all brain regions (from 56% in the neurohypophysis to 94% in the cerebellum), the relative percentage of Mr 15,000 and 3,000 forms ranged from 1.5% and 5% respectively in the cerebellum to 20% for each in the brain stem. In one region, the neurohypophysis, S-28 accounted for 40% of the total immunoreactivity. In contrast to the cerebellum

TABLE 1 : REGIONAL DISTRIBUTION OF THE THREE IMMUNOREACTIVE SOMATOSTATIN SPECIES IN THE RAT BRAIN

STRUCTURE	IMMUNOREACTIVE SOMATOSTATIN								relative proportion %		
	ng/structure*				ng/mg of wet weight						
	15K	S-28	S-14	T	15K	S-28	S-14	T	15K	S-28	S-14
HYPOTHALAMUS	2.83±0.34	14.07±1.31	42.50±3.43	59.40	0.056	0.280	0.850	1.186	4.7	23.6	71.7
CORTEX	17.52±1.54	23.00±1.38	71.0 ±2.62	111.60	0.029	0.040	0.120	0.189	15.5	21.0	63.5
BRAIN STEM	1.75±0.14	1.60±0.08	5.45±0.45	8.80	0.0087	0.0080	0.028	0.0447	19.9	18.2	61.9
CEREBELLUM	0.21±0.04	0.60±0.11	11.60±0.80	12.41	0.0008	0.0024	0.0464	0.0496	1.7	4.8	93.5
AMYGDALA	0.50±0.09	1.01±0.14	7.40±0.42	8.91	0.010	0.020	0.148	0.178	5.6	11.3	83.1
NEUROHYPOPHYSIS	0.013±0.020	0.18±0.02	0.25±0.04	0.44	0.0092	0.128	0.178	0.315	2.9	40.6	56.5

*mean ±SD for 3 different experiments.

where only small amounts of Mr 3,000 and Mr 15,000 were present (6% the total immunoreactivity), large quantities of high molecular weight forms were found both in the brain stem and the cortex (40% of total immunoreactivity).

That the different patterns of somatostatin like immunoreactivity are not spurious results arising from degradation or non specific sticking of low Mr somatostatin species to protein during the extraction, filtration, or RIA procedure, was assessed by measuring the recovery of exogenous Mr 15,000 or Mr 3,000, extracted from the cortex, and added during extraction of the cerebellum (Fig. 3). The recovery of these two forms averaged 90% of the original amount. In order to control that the various patterns were neither the result of degradation of the iodinated tracer during RIA nor sticking of S-14 during extraction or filtration, i) we measured the recovery of exogenous (^{125}I)Tyr-O-S-14 tracer added during extraction of hypothalamic tissues and found that the yield of recovery was about 95% of the original amount (Fig. 1), ii) we compared the retention time of the tracer on HPLC with and without previous incubation in the presence of an aliquot of either the Mr 15,000, Mr 3,000 or Mr 1,600 species. In all cases retention times were found similar, and iii) we verified that under our conditions the urea concentration did not modify the binding of the antigen to antibodies in the fractions where it coeluted with S-14. Finally the various Mr 15,000, 3,000 and 1,600 species recovered in the elution of the Sephadex G-50 column were tested for their ability to compete with (^{125}I)Tyr-O-S-14 binding to either 36-38 or Barbara antisomatostatin antibodies. Parallel dilution curves were obtained (Fig. 4). These criteria of immunological similarity, together with the molecular sieve and HPLC elution patterns, establish the chemical identity of the endogenous S-28 and S-14 with the synthetic standards.*

*It is assumed that both S-14 and S-28 possess the same chemical structures in all the brain regions here considered.

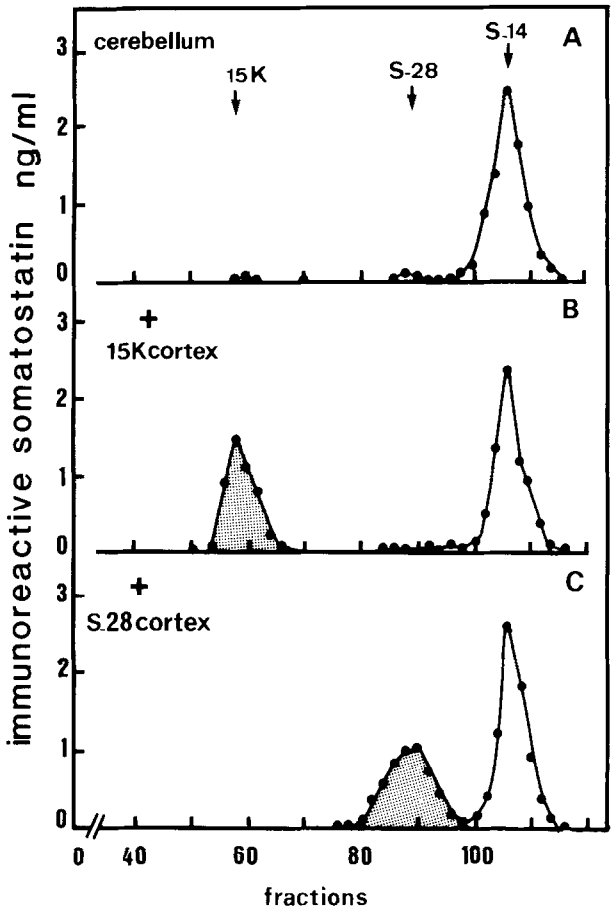

Figure 3 : *Control of the extraction yield.*

A) Elution pattern of immunoreactive somatostatin extracted from cerebellum as described in Fig. 1d.

B) Elution pattern of the same extract in which 16 ng of immunoreactive Mr 15,000 species, isolated from the cortex, were added during the initial step of extraction. Immunoreactive Mr 15,000 recovered after filtration of this extract averaged 15.2 ng.

C) Elution pattern of the same extract as in A in which 20 ng of immunoreactive S-28, originated from the cortex, were added during extraction. Immunoreactive S-28 recovered after filtration averaged 18.4 ng.

DISCUSSION :

The results presented herein demonstrate the presence of two high molecular weight immunoreactive somatostatin species (Mr = 15,000 and 3,000) in addition to S-14, both in the hypothalamus and extrahypothalamic areas of the rat. Identity of the hydrophobic, hydrodynamic and immunoreactive properties of endogenous Mr 3,000 and Mr 1,600 somatostatin species with synthetic ovine S-28 and S-14, respectively, strongly suggests close relationships between the primary sequence of these molecules and the ovine ones. The three immunoreactive forms of somatos-

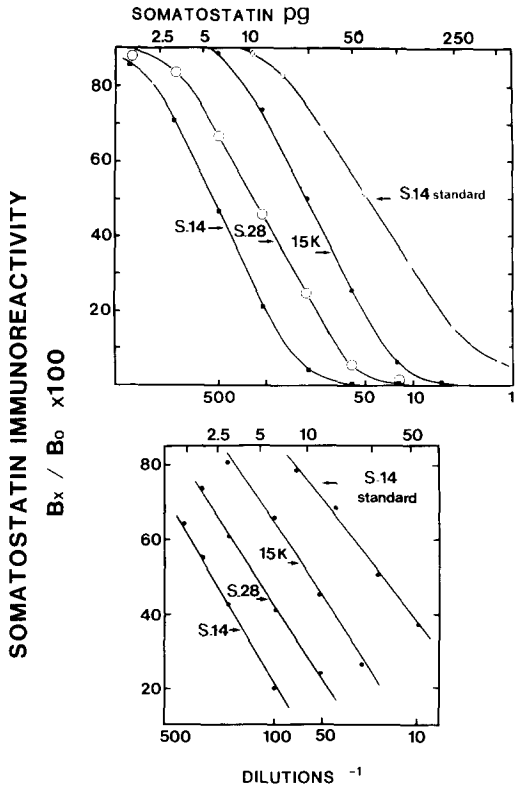

Figure 4 : *Immunological characterization of immunoreactive somatostatin like material.* Comparison of the cross reactivity of the reference somatostatin S-14 and the Mr 15,000, Mr 3,000 and Mr 1,600 species as studied by RIA using either antibodies Barbara (Top) or 36-38 (bottom). Data are expressed as Bx/Bo x 100.

tatin here detected were recognized by two antibodies exhibiting distinct sequences specificities. Parallel dilution curves were obtained with these species by reference to standard S-14. Together these observations provide indirect evidence for the presence of the S-14 sequence in the higher molecular weight forms detected in the brain extracts. They strongly suggest that the observed quantitative differences are strictly tissue specific and may have some biological significance.

The three immunoreactive somatostatin species appear to be widely distributed throughout the brain except possibly for the cerebellum. In addition, our results show that large differences in their relative percentage exist from tissue to tissue. That these differences are not the product of spurious artefacts was assessed by a number of controls. Taken together, these results indicate that the quantitative differences we observed are due neither to the action of tissue specific proteases nor to selective adsorption of endogenous somatostatin species during extraction, or to a cross-reactivity between unrelated molecules.

Of interest is the presence of S-14 in a region like the cerebellum where only very low amounts of other neuropeptides were reportedly detected (13). Indeed, these findings reinforce the hypothesis that S-14 may play a general role of neurotransmitter in various regions of the central nervous system, aside its classical, and well-documented, hormonal function. If the sole function of a precursor form is to generate, through its processing, the final active product(s), its presence in brain areas as the cortex or the brain stem in relatively high quantities may be somewhat surprising. Indeed, in those two structures, the Mr 15,000, putative prohormone, accounts for 1/5 of the total immunoreactive somatostatin. In addition, in those two structures, the two high molecular weight species (Mr 15,000 and S-28) represent together 40% of the total immunoreactivity. In contrast, the concentration gradient of Mr 15,000 , S-28 and S-14 which is observed in the cerebellum, the hypothalamus and the amygdala, is compatible with what we expected from classical precursor-final product(s) relationships. However, these results could be partly explained if the cerebellum contained predominantly somatostatin nerve endings, but the lack of conclusive immunocytochemical data concerning this structure renders this explanation highly hypothetical. Interestingly, the high percentage of S-28 in the neurohypophysis (40%), a structure known to contain exclusively nerve endings, does not argue in favor of such an hypothesis. In keeping with this feature, one can note that S-28 exhibits both a greater hormonal activity and a higher receptor binding affinity than S-14 and that the three forms of somatostatin seem to be released in the portal venous system after depolarization (14, 15). Taken together, these results reinforce the hypothesis that S-28 may play a specific biological function in various areas of both the central and periphereal nervous system.

The quantitative regional distribution of the 3 forms of somatostatin might be explained by differential intracellular processing of the Mr 15,000 putative prohormone cleavage site ($\underline{Arg}^{-1}$, $\underline{Lys}^{-2}$ from the N-terminus of the S-14 sequence). It might favour the hypothesis that S-28 could be cleaved *in vivo* into S-14 as it has been recently reported from *in vitro* experiments (16). In contrast, our results demonstrate that, in particular brain regions, this second putative precursor, S-28, represents a high percentage of the total somatostatin immunoreactivity, a finding hardly reconciliable with the above hypothesis. Therefore, the simplest model to explain differences in the regional distribution of the 3 forms of somatostatin, is to consider that the Mr 15,000 prohormone(s) could be processed at least by two different mechanisms : the first one could generate S-14 by cleavage at the prohormone site

Arg-Lys of the 15,000 sequence and/or, in particular area (for instance cerebellum) at the same site within the S-28 sequence. The second one could generate S-28 by cleavage at the Ala-Arg site (position-15, -16 upstream from the S-14 N-terminus). The differential distribution of these 3 species among the brain could reflect a modulation of these activities depending upon the tissue or the cellular localization, or both. This hypothesis implies that enzymes with distinct specificities, might be unequally distributed in those areas releasing either somatostatin-14 or somatostatin-28, or both.

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