A Discrete Thyroxine-rich Iodopeptide of 20,000 Daltons from Rabbit Thyroglobulin*

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Rabbit thyroids, labeled in vivo with 125I, were quickly removed and processed under conditions designed to minimize postmortem proteolysis. These included gentle extraction at 2°C for 20 min, the use of pepstatin and phenylmethanesulfonyl fluoride, and rapid homogenization followed by boiling. The supernatants of boiled thyroid homogenates after reduction with mercaptoethanol showed several fast iodinated bands on gel electrophoresis in sodium dodecyl sulfate; of these the most prominent were 20,000 daltons (15.2% of total 125I of homogenate supernatant), ~31,000 daltons (6.8%), and ~15,000 daltons (3.4%). These values for molecular mass and 

125I content were consistent among the thyroids of six rabbits processed individually within the same experiment. The 20,000- and ~15,000-dalton species had more than one-half of their 125I as thyroxine. The same three iodinated bands were seen with reduced extracts of thyroid, but not in unreduced samples. On basic gel electrophoresis of unreduced samples, no 125I was found other than in 19 S thyroglobulin or its polymers.

For bulk preparations, we pooled thyroids from several 125I-injected rabbits with 250 glands obtained commercially, and isolated thyroglobulin by gel chromatography. The 20,000-dalton species was purified from reduced and alkylated thyroglobulin by gel filtration, DEAE-cellulose chromatography, and preparative gel electrophoresis. It had the following features: 1) a single band on analytical gel electrophoresis; 2) a single N-terminal residue, aspartic acid; 3) a ratio of 125I to protein which was over twice that of the parent thyroglobulin; 4) a carbohydrate content of approximately 19%; 5) 63% of its 125I was in thyroxine, compared with a value of 19% for thyroglobulin; 6) it contained 39% of the 125I-labeled thyroxine of thyroglobulin and 18% of the 125I-labeled thyroxine (the high thyroxine content was confirmed by radioimmunoassay); and 7) its molar ratio to 660,000-dalton thyroglobulin was close to one.

We conclude that rabbit thyroglobulin contains a discrete iodopeptide of 20,000 daltons which is not the product of postmortem proteolysis. It appears to be held to the larger part of the thyroglobulin molecule by disulfide bonds. Its high thyroxine content indicates an important role for it in the production and/or release of thyroid hormones.

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Thyroglobulin, a thyroidal glycoprotein of approximately 660,000 daltons, is the matrix on which the thyroid hormones thyroxine and triiodothyronine are formed. Their synthesis occurs after iodination of tyrosyl residues to form the precursors 3-iodothyrosine and 3,5-diiodothyrosine, and this process appears to take place wholly within the thyroglobulin molecule (1). Several lines of evidence suggest that the molecular structure of thyroglobulin is important to hormone formation. These include thyroglobulin's greater ability, relative to that of other proteins, to form thyroxine from iodotyrosines on in vitro iodination (1), and the finding of a limited amino acid environment surrounding thyroxine in iodopeptide fragments produced by protease digestion in vitro (2).

An understanding of thyroglobulin's role in hormone synthesis demands a detailed description of its structure, particularly that of its subunits. This has been difficult to obtain because of the protein's large size, the existence of established differences among animal species, and the occasional presence of contaminating proteolytic activity during isolation procedures. In the present paper, using rabbit thyroids, we have selected preparative methods which minimize postmortem proteolysis. We have found three iodopeptide fractions of small molecular size, two of which have most of their iodine in the iodothyronines. One of these has been isolated as a discrete glycopeptide of 20,000 daltons, and some of its properties are described.

**Experimental Procedures**

**Gel Electrophoresis**—Analytical gels were run in a pH 8.9 buffer or in 0.1% SDS1 at porosities ranging from 4% to 14%, as previously described (3, 4). Additional gels were run in 0.1% SDS and 5% urea (5). A number of molecular weight markers were used, most commonly myoglobin, human growth hormone, ovalbumin, bovine serum albumin, and its cross-linked polymer (Sigma Chemical Co.). Gels were stained with Coomassie blue R-250 for SDS gels or G-250 for boils water. Analyses of Iodine and Iodoamino Acids—125I was counted in a

1 The abbreviations used are: SDS, sodium dodecyl sulfate; 20K, the 20,000-dalton iodopeptide derived from thyroglobulin; dansyl, 5-dimethylaminonaphthalene-1-sulfonfyl.
calculated from them. For the distribution of \(^{125}\)I among the iodoamino acids, we digested protein or peptide fractions with pronase (Calbiochem), separated their iodoamino acids by descending paper chromatography in butanol:2 N NH₄OH (5:1:2), and counted for radioactivity, as previously described. Occasionally, additional chromatograms were run in a butanol:2 N acetic acid system. In calculating the distribution of \(^{125}\)I among the iodoamino acids, we excluded the small amounts found at the origin and as iodide, attributing these, respectively, to undigested iodopeptides and deiodinated iodoamino acids. Together these were usually less than 10% of the \(^{125}\)I on the chromatogram.

The thyroxine content of the 20,000-dalton iodopeptide was also assessed by radioimmunoassay after pronase digestion, using a double antibody system similar to that of Chopra (8), described in detail elsewhere (9).

**Amino Acid Analyses—** Samples were hydrolyzed in constant boiling HCl at 105°C for 36 and 72 h in sealed, evacuated tubes, and measured on an automatic amino acid analyzer (Dionex, Sunnyvale, CA) connected to an integrator (Supergrator I, Columbia Scientific Co.). The recommended Dionex program was slightly altered to identify homosamin and tryptophan. For tryptophan we hydrolyzed in 3 N mercaptoethanesulfonic acid (10) instead of HCl.

**NH₄ Terminal Analyses—** Samples were reacted with dansyl chloride, followed by hydrolysis in constant boiling HCl at 105°C in sealed, evacuated tubes. We identified the dansyl amino acids by two- and three-dimensional thin layer chromatography, using the method of Percy and Buchwald (11), as previously described (3).

**Other Analyses—** The protein content of samples was measured by the method of Lowry et al. (12) using bovine serum albumin as standard. We estimated the total carbohydrate content by the alkaline ferricyanide method of Krystal and Graham (13), using their correction factors for hydrolytic destruction. Cathepsin D activity was measured by the release from hemoglobin of peptides soluble in trichloroacetic acid (14).

**Disulfide Bond Cleavage—** Samples were reduced with mercaptoethanol, 50 mol per mol of disulfide bond, at 25°C for 4 h, and alkylated with acrylonitrile (15), 2 mol per mol of mercaptoethanol, for 30 min at 25°C, pH 8.0. The reaction was stopped with mercaptoethanol and excess reagents removed by dialysis against water or by gel filtration. In the samples used for amino acid analyses, we alkylated with iodoacetic acid (16) or oxidized thyroglobulin with performic acid (17).

**Animals—** All isotopic experiments were conducted with young female New Zealand White rabbits, maintained on a standard diet of laboratory chow containing approximately 0.8 \(\mu\)g of iodine per g.

**Preparation of Thyroid Extracts—** Three rabbits were killed 6 days after each received an intraperitoneal injection of 0.5 mCi of carrier-free Na\(^{125}\)I. The thyroids were removed quickly, cut into three slices per lobe. The pooled slices were placed either into 0.06 M sodium phosphate buffer, pH 7.0, or into the same buffer plus the proteolytic enzyme inhibitors pepstatin \((10^{-5} \text{ M})\) and phenylmethylsulfonyl fluoride \((10^{-3} \text{ M})\), and extracted at 2°C for 20 min, followed by centrifugation at 122,000 \(\times g\) for 35 min at 4°C. Portions of the extract were placed immediately on polyacrylamide gels for electrophoresis. Other aliquots were first made 1% SDS, with or without mercaptoethanol, and run on gels in SDS.

**Preparation of Boiled Thyroid Homogenates—** Six rabbits were killed 3 days after each had an intraperitoneal dose of 0.5 mCi of Na\(^{125}\)I. Each thyroid was removed quickly, cut into three slices per lobe, reduced with mercaptoethanol, 50 mol per mol of disulfide bond, at 25°C for 4 h, and alkylated with acrylonitrile, 2 mol per mol of mercaptoethanol, for 30 min at 25°C, pH 8.0. The reaction was stopped with mercaptoethanol and excess reagents removed by dialysis against water or by gel filtration. In the samples used for amino acid analyses, we alkylated with iodoacetic acid or oxidized thyroglobulin with performic acid.

**Preparation of 20K by gel filtration.** Column (4.4 × 86 cm) was packed with 6% agarose (Bio-Gel A-5m) equilibrated with 0.06 M sodium phosphate, pH 7, in 0.1% SDS. S-Cyanoethylated thyroglobulin (750 mg) was placed on column and fractions of 10 ml collected, at a flow rate of 39 ml per h. Aliquots were counted for \(^{125}\)I. Fractions in the shaded area were pooled and used for further purification.

**Preparation of 125I-labeled Thyroglobulin—** Seven rabbits were each given 1.1 mCi of Na\(^{125}\)I intraperitoneally and killed 3 days later. Their thyroids were removed, pooled, homogenized in 0.05 M sodium phosphate, pH 7.0, and centrifuged. The supernatant was placed on a column (1.5 × 90 cm) of Bio-Gel A-5m at 4°C in the same phosphate buffer made 0.02% with sodium azide. Thyroglobulin eluted as a single peak of \(^{125}\)I. Fractions containing it were pooled, dialyzed, and lyophilized. We prepared \(^{125}\)I-thyroglobulin from animals of the same sex and breed (Pel-Freez, Rogers, AR) by slicing and homogenizing 250 frozen thyroids and separating thyroglobulin on a similar column of A-5m (4.4 × 90 cm) followed by dialysis, lyophilization, and peeling with the \(^{125}\)I-labeled thyroglobulin. The A-5m gave a wide separation of the thyroglobulin peak from that of cathepsin D activity, and the isolated thyroglobulin showed no detectable cathepsin D activity.

In addition, we made two other preparations of \(^{125}\)I-labeled thyroglobulin by a similar procedure, but with the labeled and unlabeled thyroids pooled with each other prior to homogenization.

**Isolation of 20K—** Thyroglobulin labeled with \(^{125}\)I was reduced and alkylated, and its components partially purified by gel filtration on a column of Bio-Gel A-5m in SDS (Fig. 1). Fractions were analyzed by
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$^{125}$I content and by gel electrophoresis in SDS. The peak of $^{125}$I at tube 95 in Fig. 1 contained most of the 20K as well as smaller amounts of the ~15,000-dalton iodopeptide and unidentified heavy components. The fractions comprising this peak (the shaded area in Fig. 1) were pooled, dialyzed against water for 24 h, lyophilized, dissolved in NHHCO$_3$ in 6 M urea, 3.0 mmho, pH 8.8, and further fractionated on a column of DEAE-cellulose (Whatman DE52) (Fig. 2). From analytical gels, the material eluting at 8.8 mmho had most of its stain for protein in the 20,000-dalton zone, with traces of both heavier and lighter components. Fractions comprising this peak were pooled, dialyzed, and lyophilized. An aliquot was then dissolved in 2% SDS/S04\(\cdot\)H$_2$O in 6 M urea, 3.0 mmho, pH 8.8, and further fractionated by preparative gel electrophoresis. A center strip was cut from this preparative gel, and 5-mm segments were counted for $^{125}$I (Fig. 3), showing a major band in the 20,000-dalton area (the shaded area in Fig. 3). The remainder of this band was then homogenized in water, centrifuged, and the supernatant dialyzed and lyophilized.

We used this preparation of 20K, referred to as Preparation I, for the analytical studies recorded below, unless otherwise specified. Preparation II was isolated in a similar manner from the same thyroglobulin sample alkylated with iodoacetic acid instead of acrylonitrile. It was used particularly for analyses of half-cystine and glutamic acid, since S-cyanoethyl cysteine elutes with glutamic acid on the amino acid analyzer. Preparations III and IV were made from S-cyanoethylated thyroglobulin by methods similar to those of Preparation I, but with labeled and unlabeled thyroid homogenates pooled before thyroglobulin isolation. Preparation V was oxidized with periodate prior to isolation of 20K.

RESULTS

Thyroid Extracts—On basic gels of 4% polyacrylamide, thyroid extracts with or without inhibitors showed over 85% of the $^{125}$I in a slow band (Fig. 4, left panel). This band was identical in position with that of 19 S thyroglobulin, run on parallel gels and identified by analytical ultracentrifugation in a previous study (2). The only other $^{125}$I-containing band corresponded to 27 S thyroglobulin. There was no iodination of albumin or other small molecules.

On 4% gels in SDS, most of the $^{125}$I was at the origin or in a slow band of ~290,000 daltons (Fig. 4, center panel). When the sample was reduced with mercaptoethanol, several new bands appeared, including one of ~20,000 daltons (8.2% of the total $^{125}$I on the gel), ~15,000 daltons (2%), and ~37,000 daltons (1.6%) (Fig. 4, right panel). A similar pattern was seen for the extract without enzyme inhibitors, in which the 20,000-dalton band contained 8.1% of the $^{125}$I, the 15,000-dalton peak 1%, and the ~37,000-dalton peak 3.2%.

Boiled Homogenates of Thyroid—The supernatants from homogenates of each of the six rabbit thyroids showed five discrete peaks of $^{125}$I on 4% gels in SDS after mercaptoethanol (Fig. 5). Gels of 8% in SDS, both with and without 8 M urea, were also run to provide sharper peaks for the low molecular weight components. Table I shows that the six preparations were similar to one another in distribution of $^{125}$I among these peaks. The molecular sizes of the individual $^{125}$I-containing bands were estimated from standards of known molecular weight run on parallel gels, and were quite reproducible among the six homogenate supernatants. For Band B, taken from the 4% gels, the mean estimated molecular weight (± S.E.) was 233,000 ± 6,000. Bands C, D, and E, from the 8% gels, were, respectively, 32,000, 21,000, and 15,000, all with S.E. values less than 1,000.

The distribution of $^{125}$I among the iodooamino acids of Bands A to E from an individual rabbit is shown in Table II. The smaller bands contained much more of their $^{125}$I as thyroxine than the larger ones, a finding confirmed in other experiments. The thyroxine values shown for Band E are probably artifactually low. In another experiment with more of the ~15,000-dalton material available, chromatography in a butanol/acetic acid system separated an iodopeptide which had co-migrated with 3-iodotyrosine in the butanol/ethanol/ammonia system. The $^{125}$I distribution of the ~15,000-dalton iodopeptide in this latter experiment was 21% as diiodotyrosine, 8% as 3-iodotyrosine, 67% as thyroxine, and 3% as triiodothyronine. This distribution is representative of most other determinations of the ~15,000-dalton species. We do not have an explanation for the high triiodothyronine content obtained in Band E of Table II.

Isolation of 20K—Fig. 6 shows gels at various steps in the purification of 20K for Preparation I. The A-5m column after reduction and alkylation removed excess reagents and most of the heavy components of thyroglobulin. The DEAE-cellulose separated 20K from a noniodinated material of similar...
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945

I

I

0

80

0

40

0

40

80

(mm)

FIG. 5. Gel electrophoresis of the supernatant of a boiled thyroid homogenate. The abscissae show the gel length; the ordinates show the percentage of the gel's total ¹²⁵I found in each 2.5-mm segment. Samples were prepared in 1% SDS, 1% mercaptoethanol, and run on gels of 4% (top) or 8% (bottom) polyacrylamide in 0.1% SDS.

Table I

Distribution of among five bands (A to E) from electrophoresis of reduced supernatants of boiled thyroid homogenates on gels of 1% polyacrylamide in 0.1% SDS

Data from six rabbits are presented individually. Table entries are the percentage of the total of the gel found in each band. Bands are labeled as in Fig. 5.

| Band | Rabbit A | Rabbit B | Rabbit C | Rabbit D | Rabbit E |
|------|----------|----------|----------|----------|----------|
| (origin) | (-23K) | (-32K) | (-20K) | (-15K) | % of total ¹²⁵I on gel |
| 1 | 20.3 | 27.8 | 6.1 | 14.7 | 2.4 |
| 2 | 23.5 | 24.2 | 6.6 | 17.8 | 2.3 |
| 3 | 20.8 | 25.6 | 6.7 | 11.6 | 1.8 |
| 4 | 14.9 | 27.4 | 6.6 | 15.4 | 3.5 |
| 5 | 17.4 | 26.0 | 6.9 | 12.8 | 2.6 |
| 6 | 10.1 | 18.9 | 7.7 | 19.2 | 1.8 |

molecular size and from the ~15,000-dalton iodopeptide. Preparative gel electrophoresis also separated 20K from the ~15,000-dalton and ~31,000-dalton species. In the DEAE-cellulose step of Preparation III, we eluted 20K at a conductivity of 7.0 mmho, and on analytical gel electrophoresis found almost all of the stain in one major band at 20K with several faint bands visible on overloaded gels. For many purposes, isolation with the A-5m and DEAE-columns is satisfactory and avoids the limitations and losses of the preparative gel step.

Properties of 20K—Analytical electrophoresis of the purified 20K of Preparation I showed a single heavy band of 20K (Fig. 6d). Very faint traces of several slower bands were occasionally present in this and the other three preparations. In all preparations there was only one band of ¹²⁵I, corresponding exactly to the stained 20K band.

Dansylated 20K showed a single NH₂-terminal residue, aspartic acid. This result was confirmed in Preparation III as well. Measurements of the ¹²⁵I peak of 20K on SDS gels of 6%, 8%, 10%, 12%, and 14% polyacrylamide gave molecular weight estimates of 19,800, 21,300, 19,200, 20,000, and 21,200, respectively, when compared with protein markers on parallel gels. Preparation IV on a similar series of gels gave a mean value of 20,000. Gels of 4% polyacrylamide gave somewhat higher values, in keeping with the glycopeptide nature of 20K (see below) (18).

Composition of 20K—Using the conditions and correction factors of Krystal and Graham (13) for hydrolytic destruction, we found the total carbohydrate content to be 10.0% by weight. This value is close to that of intact thyroglobulin (2). This method does not measure sialic acid, which represents 1.7% of the residue weight of rabbit thyroglobulin (2), and

Table II

Distribution among the iodoamino acids of ¹²⁵I from the major iodinated bands on gel electrophoresis of the reduced supernatant from a boiled thyroid homogenate

Data are from a single rabbit (No. 3 of Table I).

| Iodoamino acid | Band |
|----------------|------|
| Diadosyltyrosine | A (origin) |
| 3-Iodothyrosine | B (~23K) |
| Thyroxine | C (~32K) |
| Triiodothyronine | D (~20K) |
| 3-Iodotyrosine | E (~15K) |

Entries are the contribution, by percentage, of each iodoamino acid to the total ¹²⁵I of the band.

Fig. 6. Electrophoresis on gels of 8% polyacrylamide in 0.1% SDS of 20K at several stages of purification. Gels are, from left to right: (a) reduced alkylated thyroglobulin; (b) after gel filtration on A-5m (shaded area of Fig. 1); (c) after DEAE-chromatography (shaded area of Fig. 2); and (d) after preparative gel electrophoresis (shaded area of Fig. 3). The direction of migration is from top to bottom. The arrow indicates the 20K band. Each gel contained approximately 15 to 30 µg of 20K.
TABLE III

Amino acid composition of the 20,000-dalton iodopeptide

| Amino acid       | Residues/mol |
|------------------|--------------|
| Aspartic acid    | 16           |
| Methionine       | 8            |
| Serine           | 11           |
| Glutamic acid    | 21           |
| Proline          | 3            |
| Glycine          | 19           |
| Alanine          | 21           |
| Half-cystine     | 5            |
| Valine           | 15           |
| Methionine       | 2            |
| Isoleucine       | 7            |
| Leucine          | 15           |
| Tyrosine         | 3            |
| Phenylalanine    | 5            |
| Histidine        | 3            |
| Tryptophan       | 1            |
| Lysine           | 8            |
| Arginine         | 8            |

TABLE IV

Specific activity and distribution of iodine in 20K and in thyroglobulin

|        | 20K         | Thyroglobulin |
|--------|-------------|---------------|
| 125I   | (cpm/ng)    | (cpm/mg)      |
|        | 5.5         | 2.5           |
| 127I/125I (ng/mg) | 17.9 | 10.2         |
| 127I/125I (cpm/mg) | 98.3 | 25.5         |

Distribution of 125I

| Iodoprotein     | 24% | 49% |
|-----------------|-----|-----|
| 3-Iodotyrosine  | 12  | 30  |
| Thyroxine       | 63  | 19  |
| Triiodothyronine| 1   | 2   |

* Entries are the contribution, by percentage, of each iodoamino acid to the total 125I of 20K or of thyroglobulin.

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Our study demonstrates that reduced rabbit thyroglobulin contains several iodinated components of small molecular weight and that they are not the products of postmortem proteolysis. Quantitatively, the most important of these components is the 20,000-dalton species. Its high thyroxine content and its high specific activity of iodine imply a preferred site for thyroxine formation, and although it comprises only about 3% of the protein's weight, it contains over one-third of the newly formed hormone. We have found a similar thyroxine-enriched peptide of 20,000 to 25,000 daltons in other species, including rat and man. The other two small iodinated bands of thyroglobulin, ~31,000 daltons and ~15,000 daltons, have not yet been extensively characterized. The very high iodothyronine content of the ~15,000-dalton component suggests that it is physiologically important.

Distribution of Iodine

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| Thyroxine       | 63  | 19  |
| Triiodothyronine| 1   | 2   |

* Entries are the contribution, by percentage, of each iodoamino acid to the total 125I of 20K or of thyroglobulin.

Actual values for thyroxine may have been higher, since its ratio of 125I to 127I is usually lower than that of the iodothyronines for a number of days after injection of Na2121.

From analytical gels, we found that 20K contained 11.8% of the total 125I of thyroglobulin in this preparation. Thus, we can derive from Table IV that it contained 39% of the 125I-labeled thyroxine of thyroglobulin and 18% of the unlabeled thyroxine. From these data we also calculate that 20K contributed 3.1% of the weight of thyroglobulin. For Preparation III, 20K represented 2.1% of thyroglobulin's weight, and for Preparation IV, 3.6%.

In Preparation IV we confirmed the chromatographic identification of thyroxine by radioimmunoassay. This gave a value of 20.2 nmol of thyroxine per mg of 20K compared with 33.3 calculated from data similar to those of Table IV for Preparation IV.

DISCUSSION

Our study demonstrates that reduced rabbit thyroglobulin contains several iodinated components of small molecular weight and that they are not the products of postmortem proteolysis. Quantitatively, the most important of these components is the 20,000-dalton species. Its high thyroxine content and its high specific activity of iodine imply a preferred site for thyroxine formation, and although it comprises only about 3% of the protein's weight, it contains over one-third of the newly formed hormone. We have found a similar thyroxine-enriched peptide of 20,000 to 25,000 daltons in other species including rat and man. The other two small iodinated bands of thyroglobulin, ~31,000 daltons and ~15,000 daltons, have not yet been extensively characterized. The very high iodothyronine content of the ~15,000-dalton component suggests that it is physiologically important.

It is not clear how these iodopeptides, particularly 20K, relate to the parent thyroglobulin. Thyroglobulin is isolated from the thyroid principally as a 19 S form of 660,000 daltons, which can dissociate into 12 S half-molecules. There is controversy over whether the two 330,000-dalton half-molecules are identical (19, 20). The 3% of thyroglobulin's weight found in 20K would represent approximately 1 mol of 20K per mol of 660,000-dalton thyroglobulin. We can consider several possibilities for interpretation of this relationship. (a) There may be 1 mol of 20K in one of two nonidentical 330,000-dalton half-molecules. (b) 20K may be a separate component of the 660,000-dalton protein in addition to, rather than as part of, the two half-molecules; its attachment to the remainder of thyroglobulin could occur by disulfide formation during iodination (21), a post-translational event. (c) There may be variability in the synthesis of thyroglobulin, with only some of the molecules containing 20K; thyroglobulin has already been found heterogeneous in its composition of iodoamino acids in two other species (4, 22) and even in samples isolated from different parts of the same thyroid (23). (d) Finally, 20K may represent an early step in the physiological, i.e. antemortem, proteolysis of thyroglobulin. Further work is necessary to permit a choice among these or other possibilities.
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