

# Lutein associated with a transthyretin indicates carotenoid derivation and novel multiplicity of transthyretin ligands

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**Abstract** Transthyretins isolated from different species bind hydrophobic compounds and are often obtained in a yellow form. Such a transthyretin from chicken serum was purified by chromatography using Sepharose-coupled human retinol-binding protein. The yellow chromophore was extracted with methanol and purified by reverse phase HPLC followed by normal-phase chromatography on a nitrile column. Ultraviolet-visible absorbance and mass spectrometry identified the yellow compound as lutein, i.e. xanthophyll, (*all-trans*)- $\beta,\epsilon$ -carotene-3,3'-diol, estimated to constitute 10–30% of associated colourless compounds. These components are different from the yellow component isolated from human transthyretin and establish that carotenoid-derived pigments can be associated with transthyretins.

**Key words:** Lutein; Carotenoid binding; Transthyretin; Yellow component; Mass spectrometry

## 1. Introduction

Human transthyretin (TTR) is known in three-dimensional structure at 1.8 Å resolution [1], occurs in many different forms [2], and is associated with other compounds, including yellow component(s) found in TTR from mammalian and avian forms of the protein [3]. Transthyretin has two well-established functions; one is the transport of thyroxine [4], the other the transport of retinol via the TTR-associated retinol binding protein (RBP) [5], but other functions have also been considered. The protein is structurally well conserved throughout evolution [6], particularly in its thyroxine-binding channel, but TTR is not essential for life as it is absent in amphibian and lower vertebrate branches [7], and as TTR-null-mutant mice survive, are euthyroid, and reproduce, although with reduced levels of triiodothyronine and thyroxine, as well as extremely low levels of retinol and RBP [8].

TTR is synthesized mainly in the liver, but also in the choroid plexus. While, the synthesis in the choroid plexus is found in species down to reptiles, that in liver is found only in mammals, one group of marsupials, and birds [6,7,9]. TTR is also produced in the  $\alpha$  cells of the human Langerhan's islets, but in the  $\beta$  cells or in both the  $\beta$  and  $\alpha$  cells in other species [10]. Furthermore, specific cells in the gastrointestinal tract produce TTR, in particular in the fetus [11], as do the pigment epithelial cells of the retina [12] and cells in the pineal gland [13]. Human TTR is involved in pathological conditions characterized by amyloid

deposits associated with both senile [14] and familial forms [15] of systemic amyloidosis. A number of TTR variants with single amino acid substitutions show increased ability to form the amyloid deposits. Yellow component(s) are present also in amyloid transthyretin [16].

This paper reports on the isolation of the yellow compound from chicken TTR and identifies it as the hydroxylated carotene derivative lutein. While other compounds may constitute yellow TTR ligands in other species, this work recognizes a novel multiplicity in TTR ligands by addition of carotenoid-derived ligands.

## 2. Materials and methods

### 2.1. Protein purification

Chicken blood was collected at an abattoir and serum was separated from the retracted blood cells without centrifugation. Outdated plasma was used as source of human TTR. For isolation of chicken RBP and TTR, respectively, Sepharose coupled with human TTR or human RBP was prepared [17]. Before isolation of TTR, RBP was eliminated from the serum by affinity chromatography on human TTR-Sepharose. A total of 40 litres of chicken serum was processed on two different occasions. Agarose electrophoresis [18], isoelectric focusing [2], and polyacrylamide gel electrophoresis under native conditions and in the presence of SDS [19] were performed as described.

### 2.2. Purification of yellow component

Purified TTR and RBP were desalted on Sephadex G-25 Fine and lyophilized. Methanol was added and the mixture was shaken for 10 min. The proteins were removed by centrifugation and the supernatants evaporated to dryness. The yellow extract from chicken TTR, the retinol extract of chicken RBP, and the extract from human TTR were stored in methanol at  $-70^{\circ}\text{C}$ . The methanol extracts were studied by capillary electrophoresis in a Beckman P/ACE 2000 instrument using a mixture of 0.05 M phosphate buffer, pH 2.5, and methanol (1:4) at 20 kV for 40 min with monitoring at 200 nm. For HPLC purification, the yellow methanol extract from chicken TTR was applied to a semi-preparative reverse phase C18 column (Nucleosil 5, 100 Å, 250  $\times$  10 mm; Phenomenex, Scandinavian Genetec, Kungälv, Sweden) eluted with a methanol gradient in water at a flow rate of 3 ml/min. The eluent was monitored at 260 and 444 nm. The yellow material eluted as a single chromatographic peak and was purified further by normal-phase HPLC on a Nucleosil 5CN nitrile column (Scandinavian Genetec) in the solvent system hexane/dichloromethane/methanol/*N,N*-diisopropylethylamine (74.65:25.00:0.25:0.10) [20]. The yellow material eluted as a single chromatographic peak and was collected for structural studies. Xanthophyll (lutein) from Sigma was purified in the same manner.  $\beta$ -Carotene was also obtained from Sigma.

### 2.3. Spectrometry

The absorption spectra of the compounds isolated from chicken and human TTR, respectively, were recorded from 200 to 600 nm in a Hitachi U3200 spectrophotometer and compared with that of the reference lutein. The molecular weight of the isolated yellow compound from chicken TTR was determined by mass spectrometry using a VG

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Auto Spec-Q instrument, equipped with a fast-atom bombardment (FAB) source and a cesium ion gun operated at an anode potential of 25 kV. The samples were dissolved in 70% methanol and 5–10  $\mu$ l were applied to the FAB target coated with a matrix of glycerol/nitrobenzyl alcohol (1:1 v/v). Positive ion spectra were recorded at an accelerating voltage of 8 kV and a resolution of 1000 (5% valley).

### 3. Results

#### 3.1. Chicken TTR

Affinity chromatography of TTR from chicken serum resulted in a product of high purity (>98%) as judged by electrophoresis (Fig. 1A). Chicken TTR presented the same type of microheterogeneity pattern as human TTR upon non-denaturing (Fig. 1B) and denaturing isoelectric focusing. The purified chicken TTR was visibly yellow at a concentration of 10 mg/ml. The protein remaining after methanol extraction was uncoloured. Upon reverse-phase HPLC, the methanol extract showed only one peak with absorbance in the visible range. However, several other peaks with absorbance below 350 nm were separable (Fig. 2A). The total amount of extractable material varied between TTR preparations, from 2% to 6% of the mass of the lyophilized material, with the yellow compound constituting 10–30% (w/w) of the extracts. A peak corresponding to thyroxine was identified (Fig. 2A), and retinol was present (Fig. 2A) because of a minor contamination of the TTR preparation with RBP (Fig. 1A). Elimination of RBP from the TTR preparation also eliminated retinol from the methanol extract. The normal phase HPLC system was capable of separating isoforms of hydroxylated carotenoids, but identified only one peak at both 260 and 444 nm, with an elution time identical to that of the lutein reference. The ultraviolet-visible spectra of the yellow compound from chicken TTR and the lutein standard were identical (Fig. 2B).

The positive ion FAB mass spectra of the reference lutein and the compound extracted from TTR showed an intense ion at  $m/z$  568 and a fragment ion at  $m/z$  551 (Fig. 3). These correspond to the radical cation of lutein and to loss of water from a protonated molecular ion, respectively. These results identify the yellow component associated with chicken TTR as the most common isomer of lutein, (*all-trans*)- $\beta$ , $\epsilon$ -carotene-3,3'-diol (Fig. 4).

#### 3.2. Human TTR

Methanol extracts of human TTR were yellow, but to a lesser extent than those chicken-derived. However, extraction of human TTR at acidic pH (~2) and elevated temperatures (100°C for 5–10 min) can result in the recovery of a highly yellow substance with absorbance maxima at 268, 282 and 430 nm (Fig. 2B), interpreted to constitute a pterin derivative [21]. Mass spectrometric studies of that material have so far been unsuccessful. However, capillary electrophoresis has resolved extracts from both chicken and human TTR into two groups of major peaks at identical positions but in different ratios.

### 4. Discussion

#### 4.1. Yellow component in chicken TTR

Most carotenoids have a typical spectrum with three strong absorbance maxima in the region 400–550 nm and weak maxima in the 260–280 and 320–350 nm regions [22]. The spectrum of purified chicken TTR shows this pattern and is identical to that of lutein. The mass spectrum also indicates that the yellow carotenoid associated with chicken TTR is lutein. The lutein content of the total methanol extract is approximately 10–30% by weight. The other components, identified by their 260 nm absorbance, elute before lutein. Mass spectra of these components indicate molecular weights between 280 and 498 Da,

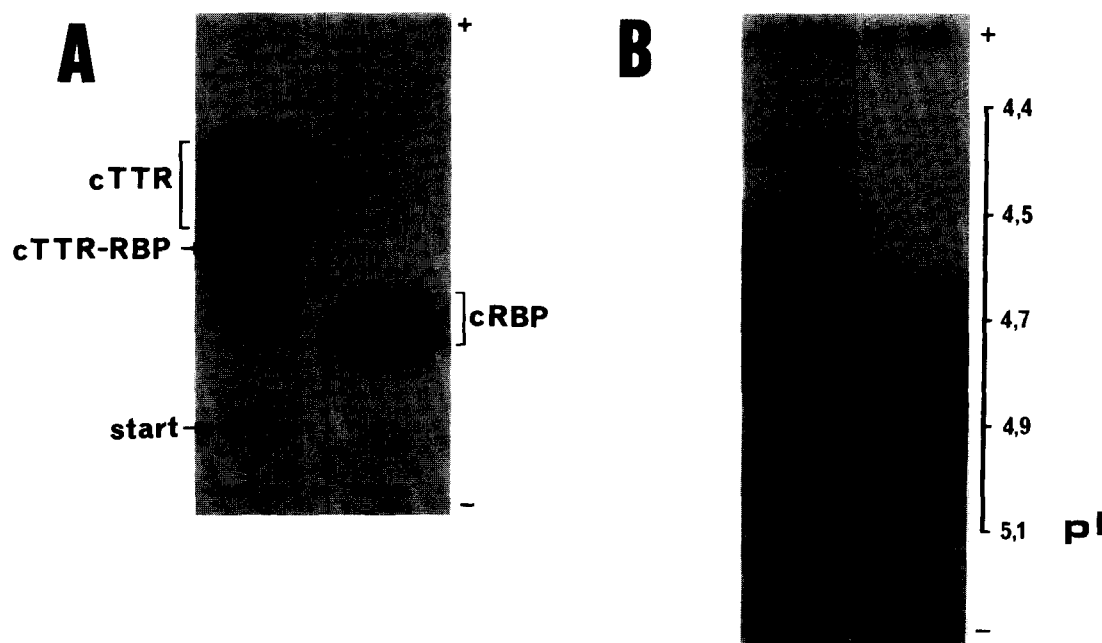


Fig. 1. Agarose electrophoresis (A) in 0.075 M barbital buffer, pH 8.6, of purified chicken TTR (cTTR, left lane), and purified chicken RBP (cRBP; right lane) after one step of purification on Sepharose-human RBP and Sepharose-human TTR, respectively. Isoelectric focusing (B) of isolated chicken TTR (left lane) compared with that of human TTR (hTTR; right lane). The purified TTR in A is slightly contaminated with chicken RBP in TTR complex as indicated. Protein staining by amido black in A and Coomassie brilliant blue in B.

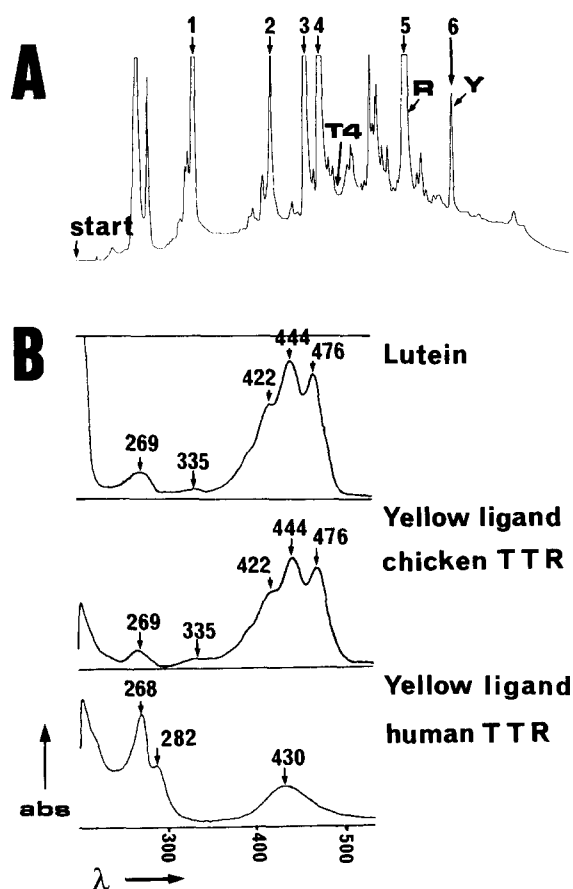


Fig. 2. Chromatogram at 260 nm (A) of chicken TTR methanol extract subjected to reverse-phase HPLC in methanol/water with a gradient from 20% to 100% methanol in 15 min, 100% methanol for 10 min and back to 20% methanol in 5 min. Peaks 1–6 represent major components, that in peak 6 is strongly yellow (Y) with three absorbance maxima as indicated in B, middle panel. The shoulder corresponding to thyroxine (T4) and the peak corresponding to retinol (R) are also indicated. In B, the top two panels show the similarity between the spectra of the chicken TTR yellow component and the lutein reference, while the bottom panel shows the difference toward the spectrum of the human TTR yellow component.

while preliminary NMR studies indicate structural similarities to lutein, suggesting that many of the components extracted from chicken TTR could be oxidized carotenoid products [22].

The extent of lutein saturation of chicken TTR was estimated to be about 50%, assuming a molar ratio of one lutein per protein tetramer as the maximum binding. This extent of saturation is much higher than that with thyroxine *in vivo* (which is about 1% for human TTR and about 0.1–0.2% for chicken TTR) but of similar magnitude as that with the retinol–RBP complex (which with TTR forms a 1:1 complex in human [23] and a 2:1 complex in chicken [24]). The differences in thyroxine and retinol–RBP binding between human and chicken TTR may relate to the association of lutein and other components to the proteins. Notably, the association of lutein is a specific finding for TTR, as no lutein could be identified in similar extracts of chicken RBP. Also, the binding seems to be specific for lutein, as none of the common hydrophobic  $\beta$ - and  $\alpha$ -carotenes were found to be associated with TTR. In human, both hydrophobic and hydroxylated carotenoids are trans-

ported mainly by lipoproteins [25], which are less abundant in chicken plasma, further illustrating the different patterns.

#### 4.2. Yellow component in human TTR

Mammalian TTRs also contain associated compounds, including a yellow component. Its absorption spectrum, stability, and chromatographic behaviour are different from those of lutein. The major compound appears to be a pterin-derivative [21], but the presence of additional, carotenoid-derived components should not be excluded. TTR synthesized in liver contributes to the transport of thyroxine to the peripheral tissues, while TTR synthesis in the choroid plexus is considered exclusively to transport thyroxine into the brain [26]. In human, thyroxine is transported bound to TTR (40%), thyroxine-binding globulin, TBG (50%) and albumin (10%) [27]. Chicken plasma contains no TBG, and about 80% of its thyroxine is transported by albumin, with less than 20% (about 4 nmol/l) by TTR, although the concentrations of TTR are similar in chicken and human plasma (unpublished observation). Hence, thyroxine binding to TTR is significantly lower in chicken than human plasma. Independent of exact function, the ligands now reported may contribute an explanation to the multiplicity of TTR forms and functions, as well as to the species-specific regulatory pattern of TTR in thyroxine binding. Interestingly,

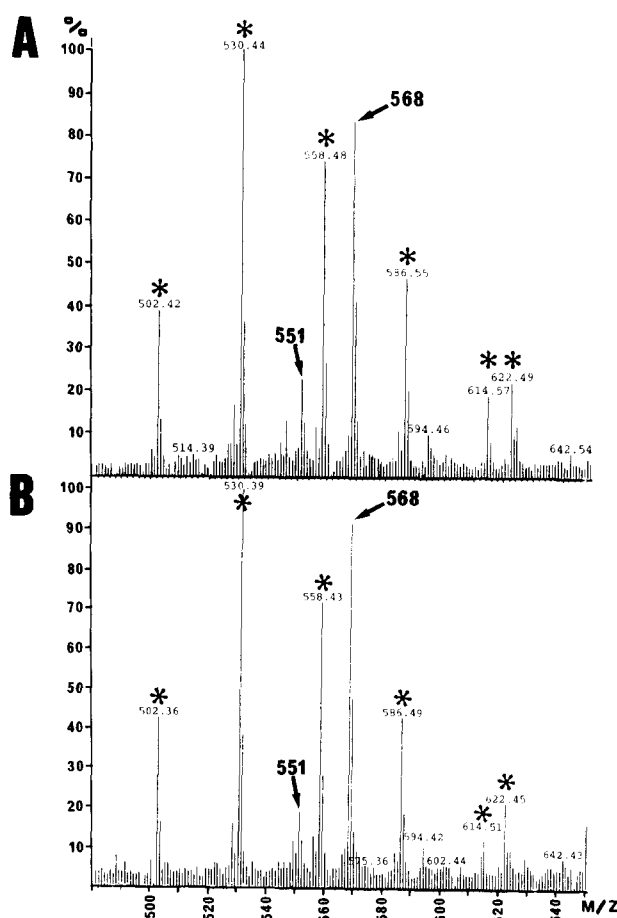
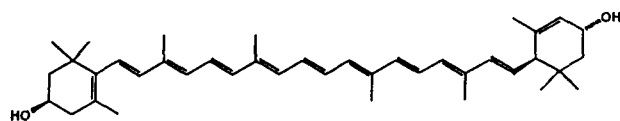


Fig. 3. Positive ion FAB mass spectra of reference lutein (A) and the yellow ligand extracted from chicken TTR (B), both purified by reverse-phase HPLC. The ions at  $m/z$  568 and  $m/z$  551 correspond to lutein derivatives (radical cation, and loss of water from a protonated molecular ion, respectively). Peaks marked \* originate from the matrix.



## Xanthophyll

### (all-E)-Lutein

### (all-E,3R,3'R,6'R)- $\beta$ - $\epsilon$ -Carotene-3,3'-diol

Fig. 4. Structure of the lutein isoform associated with chicken TTR.

the major coloured compounds of the retina are lutein and its isoform zeaxanthin [28].

Yellow and uncoloured components are present also in TTR associated with human familial amyloidotic cardiomyopathy [16], characterized by deposition of mutationally altered TTR and its fragments as amyloid fibrils. Hence, the association of ligands, including lutein and other carotenoids, is of pathophysiological interest.

#### 4.3. Conclusion

The specific binding of lutein to chicken TTR recognizes carotenoids as TTR ligands. Together with previous conclusions on pterin binding to human TTR this emphasizes multiple transport functions of transthyretins. In particular, the findings offer an explanation for the disputed role of TTR in the retina and for the presence of ligands in disease-related amyloid deposits of TTR.

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