# The influence of dietary vitamin A on triiodothyronine, retinoic acid, and glucocorticoid receptors in liver of hypothyroid rats

BY M. COUSTAUT, V. PALLET, H. GARCIN AND P. HIGUERET

*Laboratoire de Nutrition – ISTAB, Université Bordeaux I, Avenue des Facultés, 33405 Talence-Cedex, France*

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**The properties of nuclear receptors belonging to the superfamily of receptors acting as transcription factors are modulated by nutritional and hormonal conditions. We showed recently that retinoic acid (RA) restored to normal the expression of receptors attenuated by hypothyroidism. The present study was designed to find out whether dietary vitamin A (as retinol) had the same effect. Propylthiouracil in drinking water induced both hypothyroidism and a vitamin A-deficient status in rats. The maximum binding capacity ($C_{max}$) of triiodothyronine nuclear receptors (TR) was unchanged, while that of nuclear RA receptors (RAR) and nuclear glucocorticoid hormone receptors ($GR_n$) was reduced in the liver of these hypothyroid rats. The reduced $C_{max}$ of RAR stemmed from a lower level of RAR mRNA, while the reduced $C_{max}$ of $GR_n$ was assumed to be due to reduced translocation of the receptor from the cytosol to the nucleus. Feeding the hypothyroid rats with a vitamin A-rich diet did not restore the $C_{max}$ of either RAR or $GR_n$ to normal. The lack of effect of dietary retinol on RAR expression may be attributed to either genomic (unoccupied TR block the expression of RAR genes) and/or extragenomic (hypothyroidism decreases the availability of retinol and/or its metabolism to RA) mechanisms. Triiodothyronine is thought to favour the translocation of glucocorticoid hormone receptors from cytosol to nucleus. These findings provide more information on the relationship between vitamin A and hormonal status, showing that a vitamin A-rich diet is without apparent effect on the expression of nuclear receptors in hypothyroid rats.**

**Retinol: Nuclear receptors: Hormones**

The nuclear receptors of triiodothyronine (TR), retinoic acid (RAR), and glucocorticoid hormone (GR) belong to a superfamily of receptors which act as transcription factors (for review see Tsai & O'Malley, 1994). TR and RAR are represented by several isoforms encoded by corresponding genes. Studies performed mainly with transformed cell lines have shown that these receptors are subject to both homologous regulation (regulation of a receptor by its own ligand) and heterologous regulation (regulation by a ligand which is not its own ligand). Some *in-vivo* studies have also found evidence for heteroregulation of these nuclear receptors. In rat liver, vitamin A or retinoic acid (RA) modulates the expression of TR (Higueret *et al.* 1989; Pailler-Rodde *et al.* 1991*b*) and GR (Audouin-Chevallier *et al.* 1993, 1995). Hypothyroidism leads to a reduced maximum binding capacity ($C_{max}$) of RAR (Pallet *et al.* 1994), which is also modulated by glucocorticoid status (Pallet *et al.* 1996). In a previous study (Pallet *et al.* 1994) we showed that administration of RA to hypothyroid rats restored the $C_{max}$ of RAR to normal and that treatment of hypothyroid rats with a combination of triiodothyronine ($T_3$) and RA induced a higher elevation of RAR $C_{max}$ than that observed after administration of $T_3$ alone. RA appeared to potentiate the effect of $T_3$ on the $C_{max}$ of RAR. We also showed (Audouin-Chevallier *et al.* 1995) that administration of RA to control rats enhanced translocation of

GR from the cytosol to the nucleus in liver cells. Indeed, GR are located in both cytosol (GRC) and nucleus ($GR_n$), but only $GR_n$, acting as a transcription factor, regulates gene activity. Although GR appears to shuttle between cytosol and nucleus, the mechanism responsible for localization of GR remains to be elucidated (Guiochon-Mantel & Milgrom, 1993; Akner *et al.* 1995).

The present study was designed to find out whether a vitamin A-rich diet could induce similar effects to those of RA administration on the expression of nuclear receptors in hypothyroid rats. Receptor properties were evaluated by an isotopic displacement analysis. Receptor mRNA was also quantified in order to discriminate between transcriptional and post-translational mechanisms for the alteration in receptor binding capacity.

## METHODS

### *Experimental design*

Official French regulations for the care and use of laboratory animals were followed. Male Wistar rats weighing 80–90 g were obtained from IFFA-Credo (L'Arbresle, France), and were housed three to a cage in an air-conditioned room of mean temperature 21° and with a photoperiod that followed the seasonal pattern, varying from 12 to 13 h light/d during the experiments. The animals had free access to drinking water and to a semi-purified diet prepared for us by the 'Atelier de préparation d'aliments expérimentaux' (Institut National de la Recherche Agronomique (INRA) Jouy-en-Josas, France). The diet of the control rats contained (g/kg DM): vitamin-free casein 180, sucrose 305, peanut oil 25, rapeseed oil 25, cellulose 20, maize starch 400, salt mixture 35, vitamin mixture 10 (Atelier de préparation d'aliments expérimentaux INRA de Jouy-en-Josas Domaine de Vilvert, 78350 Jouy-en-Josas, France; ref. no. 102). After 1 week of adaptation the rats were allocated at random to the following experimental groups (six rats per group):

(1) control groups: rats received, for 3 weeks or 5 weeks, the control diet containing 0·0015 mg retinol palmitate/g (C3 and C5 rats respectively).

(2) hypothyroid groups: rats received for 3 weeks (H3) the control diet containing 0·0015 mg retinol palmitate/g and propylthiouracil (6-*n*-propyl-2-thiouracil (PTU); Sigma (Saint Quentin Fallavier, France), P 3755) in their drinking water (0·5 g/l) according to Levine *et al.* (1990); after 3 weeks of PTU treatment a group of six rats was killed (H3) while the diet and the PTU treatment continued for the other rats which were randomly allocated to the following groups.

(3) hypothyroid rats for 5 weeks (H5): rats were maintained on the control diet with PTU administration for 2 additional weeks.

(4) hypothyroid group treated with $T_3$: rats were maintained on the control diet with PTU administration and were injected (intraperitoneally) daily, for 2 weeks, with $T_3$ (3,5,3′-triiodothyronine; Sigma, T 2752) in 0·05 M-NaOH at a dose of 500 $\mu$g/kg body weight (H5 + $T_3$ rats).

(5) hypothyroid group fed on a vitamin A-rich diet: rats, maintained on PTU administration, received, for 2 weeks, a diet similar to that of control rats but containing 0·06 mg retinol palmitate/g (H5 + A rats);

(6) hypothyroid group treated with $T_3$ and fed on a vitamin A-rich diet: rats, maintained on PTU administration, were injected with $T_3$ and fed on a vitamin A-rich diet for 2 weeks (H5 + A + $T_3$ rats).

The rats (not fasted) were killed by decapitation (between 08.00 and 10.00 hours) according to the following schedule: C3 and H3 rats were killed on the same day; rats constituting the other groups were killed on two days, three rats of each group being killed on the same day. The collected blood was centrifuged for 15 min at 1000 ***g*** and the serum

stored at −20° until used. The livers were rapidly excised and washed (twice) in cold saline (9 g NaCl/l) solution. A portion of liver was frozen in liquid $N_2$ and stored at −80° for mRNA quantification; the remainder was used for receptor binding studies.

### *Hormone binding*

*Isolation of liver nuclei.* All tissue fractionations were carried out at 4°. Nuclei were prepared according to the method of De Groot & Torresani (1975). A portion of liver was homogenized in 0·32 M-sucrose plus 1 mM-$MgCl_2$ (0·32 SM), filtered through cheesecloth and centrifuged at 1000 *g* for 10 min. The crude pellet was washed once and then centrifuged through a layer of sucrose (2·2 M-sucrose plus 1 mM-$MgCl_2$) at 100000 *g* for 60 min. The nuclear pellet was gently resuspended in 0·32 SM plus 2·5 ml/l Triton X-100, centrifuged at 1000 *g* for 10 min and washed once with 0·32 SM.

*Triiodothyronine binding.* The final nuclear pellet derived from 2 g liver was gently resuspended in 2·66 ml TKEM (20 mM-Tris-HCl, 0·4 M-KCl, 2 mM-EDTA and 1 mM-$MgCl_2$; pH 7·9, 25°). After 30 min at 0° with frequent pipetting of the suspension to disrupt nuclei, the nuclear residue was pelleted by centrifugation at 100000 *g* for 30 min. The supernatant fraction which contained nuclear proteins, was used for assay of $T_3$ binding (Torresani & De Groot, 1975).

Incubations of nuclear proteins were performed in 0·2 ml TKEM containing 50 $\mu$g protein, 0·006–0·12 pmol [$^{125}$I]$T_3$ for 3 h at 20°. Because fresh liver tissue was used for obtaining nuclear protein dithiothreitol (DTT) was not added to the incubation medium. Indeed since DTT is known to enhance $T_3$ binding it may have minimized the differences of affinity between the experimental groups of rats. Thus the affinities measured are lower than those usually reported. The binding reaction was stopped by the addition of 1·8 ml of an ice-cold Dowex IX8-400 resin (Sigma, St. Louis, MO, USA) suspension in TKEM (40 mg/ml). After mixing, the resin was sedimented by centrifugation (1000 *g*, 5 min).

Estimation of protein-bound $T_3$ was made by measuring radioactivity in a portion of the supernatant fraction. Non-specific $T_3$ binding was determined by incubation in the presence of a 1000-fold excess of unlabelled $T_3$. All incubations were performed in duplicate. Saturation curves and Scatchard analysis were performed using final concentrations of [$^{125}$I]$T_3$ in the incubation medium ranging from 0·03 to 0·6 nM.

*Retinoid binding.* Due to the sensitivity of RA to numerous physiochemical factors (particularly to light and $O_2$) and its rapid degradation by enzymes contained in tissue extracts, a synthetic analogue of RA was used as ligand. This analogue, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl) benzoic acid (CD367), was synthesized and tritiated at the Centre International de Recherche Dermatologique (CIRD Galderma, Sophia Antipolis, Valbonne, France). CD367 behaves as a non-selective high-affinity ligand for the three types of RAR (RAR$\alpha$, RAR$\beta$ and RAR$\gamma$; Delescluse *et al.* 1991). Previous experiments performed in our laboratory have validated the use of CD367 to study binding of RAR in rat liver (Audouin-Chevallier *et al.* 1993).

To obtain RAR the nuclei were washed three times with binding buffer (10 mM-HEPES, 1·5 mM-$MgCl_2$, 10 mM-KCl, pH 7·9) and then submitted to a DNase I (Sigma no. D 4527) digestion for 30 min at room temperature, followed by a high-salt extraction (0·5 M-NaCl). The nuclear extract was then obtained by centrifugation at 12000 rev./min for 5 min. The measurement was performed according to Daly *et al.* (1990). Portions of nuclear extract (96 $\mu$l) were mixed with 4 $\mu$l of increasing concentrations (0·0125–0·125 $\mu$M in dimethyl sulphoxide) of CD367 ($195{\cdot}36 \times 10^{10}$Bq/mmol). After 1 h incubation at 4°, 50 $\mu$l of the incubation mixture was submitted to high-performance size-exclusion chromatography separation on a TSK gel G3000SW column (300 × 7·5 mm, Tosho Haas Stuttgart, Germany), and eluted with 0·3 M-$KH_2PO_4$, pH 7·8, at a flow rate of 0·5 ml/min.

The column was calibrated with a mixture of human albumin (67 kDa), egg albumin (45 kDa), and horse myoglobin (16·8 kDa). Fractions of 0·2 ml were collected and counted in a liquid scintillation counter (Beckman LS 6000 SC scintillation counter; Beckman, Gagny, France) using 4 ml of Ready Safe Cocktail (Beckman) as the scintillation liquid. Radioactive counts obtained in fractions containing the RAR–[$^3$H]CD367 complex were added and expressed as picomoles of bound ligand per mg protein. Non-specific binding was determined by incubation in the presence of 1000-fold excess of unlabelled CD367.

*Nuclear glucocorticoid receptor binding.* This was carried out according to Kaufman & Shaper (1984) with slight modifications. Nuclei were suspended in STM buffer (250 mM-sucrose, 50 mM-Tris-HCl (pH 7·4 at 4°), 5 mM-$MgSO_4$). Duplicate 0·1 ml portions of this suspension were incubated overnight (16–20 h) at 4° with a range of labelled [$^3$H]dexamethasone concentrations (10–100 nmol/l) either alone, or in the presence of a 1000-fold excess of unlabelled dexamethasone to determine non-specific binding. After incubation, samples were diluted with 2 ml STM buffer and centrifuged at 1300 ***g*** for 15 min. The pellets were washed three times with 3 ml STM buffer to remove unbound hormone. Then samples were extracted with 1 ml ethanol at 22° for a minimum of 30 min, cooled to 4°, and sedimented at 1300 ***g*** for 15 min. Portions of supernatant fraction of 500 $\mu$l were counted in a liquid scintillation counter (Beckman LS 6000 SC scintillation counter) using 4 ml of Ready Safe Cocktail (Beckman) as the scintillation liquid.

*Scatchard analysis.* Scatchard curves were drawn using a linear regression analysis of the data (SigmaPlot Scientific Graphing System1 4.02, Jandel Scientific GmbH, Erkrath, Germany). The slope of the straight line gave the affinity constant (*Ka*) and the intercept of the slope with the abscissa represented the maximum binding capacity ($C_{max}$), i.e. the maximal concentration of binding sites.

### *Quantification of mRNA*

mRNA was quantified by reverse transcription and amplification by the polymerase chain reaction (RT-PCR). The values of RAR, TR and GR mRNA were obtained by comparison with the level of an internal standard, $\beta$-actin, that was simultaneously reverse-transcribed and amplified in the same test tube. $\beta$-Actin is known to be insensitive to nutritional and hormonal conditions and $\beta$-actin mRNA was previously used as the endogenous standard for semi-quantitative analysis (Mitsuhashi & Nikodem, 1989; Ma *et al.* 1990). Moreover, according to a competitive RT-PCR method (Siebert & Larrick, 1993) and using the PCR MIMIC™ Construction Kit (Clontech Laboratories, Palo Alto, USA) we verified the constancy of the level of $\beta$-actin mRNA among the experimental conditions studied.

Extraction of RNA was performed according to the method of Chomczinski & Sacchi (1987) (modified). Rat liver (400 mg) was homogenized with 4 ml of extraction buffer (5·3 M-guanidium thiocyanate + 0·2 M-Tris-HCl, pH 7·5 + 0·04 M-EDTA) – (solution DTT + N-lauroylsarcosine, 20 g/l); 3:1, v/v) and subsequently total RNA was extracted from this homogenate with an equal volume of phenol–chloroform–isoamyl alcohol (49:49:2, by vol.).

Oligonucleotide primers used for PCR were synthesized using an Applied Biosystem Model 381A DNA synthetizer (Applied Bio System, Roissy CDG, France). The positions and sequences of the different primers are summarized in Table 1.

*Preparation of cDNA.* A 110 $\mu$g portion of total mRNA and 550 ng of each downstream primer (A2, R2, E2, G2 for $\beta$-actin, RAR, TR, and GR) were used for the reverse transcription in the presence of 11 $\mu$l reaction buffer 5 × (250 mM-Tris-HCl pH 8·3, 375 mM-KCl, 15 mM-$MgCl_2$, 50 mM-DTT), 440 U Moloney Murine Leukemia Virus reverse transcriptase, 88 U RNase inhibitor, 55 U DNase I and 120 $\mu$M of each dNTP in a total

Table 1. *Sequences of oligonucleotide primers for β-actin, retinoic acid receptor (RAR), triiodothyronine receptor (TR) and glucocorticoid receptor (GR) and sizes of amplified fragments*

| | Primers | Sequences | Complementary sites | Size of amplified fragments (base pairs) |
|---|---|---|---|---|
| β-Actin* | A1‖ | AGGATGCAGAAGGAGATTACTGCC | 2814–2837 | 222 |
| | A2‖ | GTAAAACGCAGCTCAGTAACAGTCC | 3159–3135 | |
| RAR† | R1 | CTCACTGAGAAGATCCGGAAAGCCCACC | 538–565 | 143 |
| | R2 | TTGGTGGCCAGCTCACTGAATTTGTCCC | 680–653 | |
| TR‡ | E1 | TCCTGATGAAGGTGACGGACCTGC | 1247–1270 | 118 |
| | E2 | TCAAAGACTTCCAAGAAGAGAGGC | 1364–1341 | |
| GR§ | G1 | TGAGACCAGATGTAAGCTCTCCTC | 1321–1344 | 167 |
| | G2 | AATTGTGCTGTCCTTCCACTGCTC | 1488–1465 | |

* From rat cytoplasmic β-actin gene according to the sequence of Nudel *et al.* (1983).
† From murine RAR cDNA according to the sequence of Zelent *et al.* (1989).
‡ From rat TR cDNA according to the sequence of Murray *et al.* (1988).
§ From rat GR cDNA according to the sequence of Miesfield *et al.* (1986).
‖ Primers A1 and A2 were chosen in two different exons, the size of the polymerase chain reaction products provided a check that the amplified fragment was not derived from genomic DNA.

volume of 55 μl. Synthesized cDNA was then amplified by the polymerase chain reaction (PCR) technique using Taq polymerase (Saiki *et al.* 1988).

*Polymerase chain reaction analysis.* cDNA (15 μl) was used for amplification performed in a Perkin Elmer/Cetus thermocycler (Perkin Elmer France, Saint Quentin en Yvelines, France). The reaction mixture (180 μl) contained 1 μg of each primer A1, A2, E1, E2, R1, R2, G1, G2, 10 μM-Tris-HCl pH 8·5, 50 mM-KCl, 2 mM-$MgCl_2$, 10 mg/l gelatin, 0·2 mM of each dNTP, 1·85 MBq deoxycytidine-5′-triphosphate (specific activity > 111 TBq/mmol, Amersham) and 1·25 U Taq polymerase. The reaction was carried out for a total of thirty-four cycles. The cycle times were as follows: denaturation, 1 min at 95°; annealing, 1 min at 60°; primer extension, 2 min at 72°.

For quantititative analysis of PCR products, 8 μl PCR reaction mixture was withdrawn after each (from 10th to 29th) amplification cycle (Ozawa *et al.* 1990) and the coamplified fragments were separated by electrophoresis on a 10% acrylamide gel. The incorporated radioactivity was visualized by autoradiography, the bands were excised from the gel to equal rectangles and quantified by scintillation counting.

### *Assays*

Proteins were determined according to Bradford (1976) using a Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Serum vitamin A was assayed by HPLC (Leclercq & Bourgeay-Causse, 1981). Serum $T_3$ was assayed by a radioimmunoassay using a $T_3$-Amerlex-M RIA kit (Kodak Clinical Diagnostics, France).

### *Statistical procedure*

After the homogeneity of variances was verified, a one-way ANOVA was performed. Then, since there were the same number of replicates, the least significant differences (LSD) were calculated at $P < 0{\cdot}05$ and these values were tabulated together with the means and pooled standard errors (SEM).

Table 2. *Effect of hypothyroidism alone or after treatment with triiodothyronine ($T_3$) or vitamin A (A) or a combination of the two on serum levels of triiodothyronine and retinol in rats**

(Mean values for six rats, with their pooled standard error)

| Status of rats... | Control (C5) | Hypothyroid (5 weeks) (H5) | H5+$T_3$ | H5+A | H5+A+$T_3$ | Pooled SEM | LSD |
|---|---|---|---|---|---|---|---|
| Serum $T_3$ ($\mu$g/l) | 0·58[a] | 0·31[b] | > 50 | 0·36[b] | > 50 | 0·04† | 0·12† |
| Serum retinol ($\mu$g/l) | 424[a] | 294[b] | 268[b] | 242[b] | 278[b] | 24·7 | 77·5 |

LSD, least significant difference at $P < 0{\cdot}05$.
[a, b] Mean values within a row with unlike superscript letters were significantly different, $P < 0{\cdot}05$.
* For details of diets and procedures, see pp. 296–297.
† The serum $T_3$ values > 50 $\mu$g/l were not taken into account for statistical analysis.

## RESULTS

### *Serum levels of triiodothyronine and retinol*

Following 3 or 5 weeks of PTU administration in drinking water, serum levels of $T_3$ and retinol were decreased by 45% and 30% respectively relative to levels in control rats. Feeding the hypothyroid rats with a diet enriched in vitamin A did not alter these two variables significantly. $T_3$ administration, either alone or in combination with a vitamin A-rich diet, markedly increased serum levels of $T_3$ but was without effect on serum levels of retinol (Table 2). These results are consistent with the description of a vitamin A-deficient status in hypothyroid rats (vitamin A decreased in both liver and kidney) (Moore, 1957). Moore and co-workers showed that thyroid hormones are required for the intestinal conversion of carotene into retinol and the subsequent absorption of retinol. We found that feeding hypothyroid rats with a vitamin A-enriched diet (40-fold that in normal diet) failed to raise serum levels of retinol significantly. Thus vitamin A status does not appear to be corrected in hypothyroid animals by feeding them on a diet enriched in vitamin A.

### *Properties of retinoic acid receptors and quantification of retinoic acid receptor mRNA*

Only the RAR$\beta$ mRNA was quantified, as RAR$\beta$ is the predominant RAR isoform in the liver. The abundance of mRNA could thus be compared with the nuclear binding capacity of the receptors determined by the isotopic displacement analysis. After 3 or 5 weeks of PTU-induced hypothyroidism both RAR mRNA and $C_{max}$ were decreased, whereas the *Ka* of the receptors was unchanged (Table 3). Feeding hypothyroid rats with a vitamin A-rich diet did not affect the $C_{max}$, while the abundance of RAR mRNA was increased to a value not different from that of the control value. However, both RAR $C_{max}$ and mRNA were increased after administration of $T_3$ to hypothyroid rats, and further increased after a combination of $T_3$ and vitamin A. Receptor *Ka* was reduced after administration of $T_3$ and increased after the vitamin A-rich diet was given.

### *Properties of triiodothyronine receptors and quantification of triiodothyronine receptor mRNA*

The mRNA coding for proteins which bind $T_3$, i.e. c-*erbA* $\alpha$ mRNA and c-*erbA* $\beta$ mRNA, were quantified to enable comparison with the nuclear binding capacity of receptors. Following 3 or 5 weeks of PTU administration the $C_{max}$ of the TR and the amounts of TR mRNA were not significantly different from control values (Table 4). Feeding the

Table 3. *Expression of nuclear retinoic acid receptors (RAR) in the liver of hypothyroid rats after administration of triiodothyronine ($T_3$), a vitamin A-enriched diet (A) or a combination of the two treatments**

(Values are means for six rats for binding characteristics and means of three determinations performed on pools of two rats for mRNA)

| Status of rats | $C_{max}$ (fmol/mg protein) | Affinity *Ka* (litres/nmol) | RAR mRNA (% β-actin mRNA) |
|---|---|---|---|
| Control (C3) | 610[a] | 1·30[a] | 6·1[a] |
| Hypothyroid (H3) | 371[b] | 1·05[a] | 5·0[b] |
| Pooled SEM | 36·0 | 0·083 | 0·27 |
| LSD | 113·5 | 0·263 | 1·05 |
| Control (C5) | 650[a] | 1·40[b] | 6·5[b] |
| Hypothyroid (H5) | 290[c] | 1·56[b] | 4·3[c] |
| H5+$T_3$ | 448[b] | 0·57[c] | 7·1[ab] |
| H5+A | 333[c] | 1·91[a] | 5·8[bc] |
| H5+A+$T_3$ | 568[a] | 0·65[c] | 8·2[a] |
| Pooled SEM | 26·3 | 0·085 | 0·40 |
| LSD | 83·0 | 0·267 | 1·58 |

LSD, least significant difference at $P < 0·05$; $C_{max}$, maximum binding capacity.

[a, b, c] Mean values within a column for each experiment, not sharing a common superscript letter were significantly different ($P < 0·05$).

* For details of diets and procedures, see pp. 296–298.

Table 4. *Expression of nuclear triiodothyronine ($T_3$) receptors (TR) in the liver of hypothyroid rats after administration of $T_3$, vitamin A-enriched diet (A) or a combination of the two treatments**

(Values are means of six rats for binding characteristics, and means of three determinations performed on pools of two rats for mRNA)

| Status of rats | $C_{max}$ (fmol/mg protein) | Affinity *Ka* (litres/nmol) | TR mRNA (% β-actin mRNA) |
|---|---|---|---|
| Control (C3) | 411[a] | 4·80[a] | 3·8[a] |
| Hypothyroid (H3) | 390[a] | 5·05[a] | 3·6[a] |
| Pooled SEM | 42·0 | 0·32 | 0·37 |
| LSD | 132·6 | 1·01 | 1·43 |
| Control (C5) | 432[b] | 4·55[b] | 3·5[b] |
| Hypothyroid (H5) | 410[b] | 3·66[b] | 3·7[b] |
| H5+$T_3$ | 764[a] | 4·27[b] | 6·0[a] |
| H5+A | 454[b] | 4·27[b] | 4·0[b] |
| H5+A+$T_3$ | 684[a] | 5·95[a] | 6·4[a] |
| Pooled SEM | 43·0 | 0·33 | 0·33 |
| LSD | 135·7 | 1·05 | 1·28 |

LSD, least significant difference at $P < 0·05$; $C_{max}$, maximum binding capacity.

[a, b] Mean values within a column for each experiment with unlike superscript letters were significantly different, $P < 0·05$.

* For details of diets and procedures, see pp. 296–298.

Table 5. *Expression of nuclear glucocorticoid hormone receptors ($GR_n$) in the liver of hypothyroid rats after administration of triiodothyronine ($T_3$), vitamin A-enriched diet (A) or a combination of the two treatments**

(Values are means of six rats for binding characteristics, and means of these determinations performed on pools of two rats for mRNA)

| Status of rats | $C_{max}$ (fmol/mg protein) | Affinity *Ka* (litres/nmol) | GR mRNA (% β-actin mRNA) |
|---|---|---|---|
| Control (C3) | 890[a] | 0·011[a] | 10·1[a] |
| Hypothyroid (H3) | 520[b] | 0·026[b] | 9·4[a] |
| Pooled SEM | 29·8 | 0·003 | 0·53 |
| LSD | 93·8 | 0·009 | 2·09 |
| Control (C5) | 920[b] | 0·018[bc] | 9·5[a] |
| Hypothyroid (H5) | 600[c] | 0·030[a] | 8·8[a] |
| H5+$T_3$ | 1680[a] | 0·013[cd] | 9·5[a] |
| H5+A | 750[bc] | 0·020[b] | 10·4[a] |
| H5+$T_3$+A | 1530[a] | 0·008[d] | 9·4[a] |
| Pooled SEM | 79·0 | 0·002 | 0·44 |
| LSD | 249 | 0·007 | 1·74 |

LSD, least significant difference at $P < 0·05$; $C_{max}$, maximum binding capacity.

[a, b, c, d] Mean values within a column, for each experiment, not sharing a common superscript letter were significantly different, $P < 0·05$.

* For details of diets and procedures, see pp. 296–298.

hypothyroid rats with a vitamin A-rich diet had no effect on the $C_{max}$ while it increased the *Ka* of these receptors. Administration of $T_3$ either alone or together with a vitamin A-rich diet induced increased receptor expression.

*Properties of glucocorticoid nuclear receptors and quantification of glucocorticoid receptor mRNA*

Hypothyroidism decreased $C_{max}$ and increased affinity of nuclear GR, but had no influence on the abundance of GR mRNA (Table 5). A vitamin A-rich diet had no effect on the expression of GR in hypothyroid rats, while administration of $T_3$ increased receptor $C_{max}$ but was without effect on the abundance of their messengers. After simultaneous treatment by $T_3$ and vitamin A an increased $C_{max}$, similar to that observed after $T_3$ treatment alone, and a decreased *Ka* were observed.

## DISCUSSION

The main side-effect of PTU administration in rats was a hypovitamin A status; serum retinol levels were 30% lower than control levels. PTU administration thus modified, either directly or indirectly, one or more steps in retinol metabolism encompassing intestinal uptake, distribution, and the synthesis and transport of retinoic acid. This effect was not corrected by feeding the hypothyroid rats with a retinol-enriched diet (40-fold normal).

*Expression of triiodothyronine receptors.*

The $C_{max}$ of TR and TR mRNA levels were unchanged after 3 or 5 weeks administration of PTU in the drinking water. Since the serum level of $T_3$ was approximately half that of the control rats, TR were not fully occupied. This result was consistent with results of a

previous study (Pallet *et al.* 1994) and those of Bernal *et al.* (1978) who found that thyroidectomy did not affect $T_3$ binding capacity in rat liver. In general, eu-, hyper-, or hypothyroid rats were found to have a similar abundance of nuclear $T_3$ receptors with no differences in affinity (Pou *et al.* 1986). The lack of effect of hypothyroidism on $T_3$ binding capacity in liver is in agreement with the results of Strait *et al.* (1990) showing that c-*erbA* $\beta$1 mRNA, which corresponds to the main TR mRNA species in liver, was not altered in rats rendered hypothyroid by administration of methimazole.

Feeding hypothyroid rats on a vitamin A-enriched diet for 2 weeks was without effect on the $C_{max}$ and the messengers of TR. In a previous study we showed that administration of RA (two 250 $\mu$g/g doses in the 24 h before killing) to hypothyroid rats was also without effect on these variables (Pallet *et al.* 1994). Thus a moderate change in vitamin A status following PTU administration or induced by intragastric intubation of a small dose of RA had no perceptible effect on the expression of TR. On the other hand, marked changes in vitamin A status induced by feeding rats for 7–8 weeks either on a vitamin A-deficient (Higueret *et al.* 1989) or a vitamin A-enriched diet (Pailler-Rodde *et al.* 1991*b*) reduced expression of TR in the liver.

Increased binding variables were observed after treating hypothyroid animals with $T_3$ for 2 weeks. The increase was assumed to stem from up-regulation of TR by $T_3$ (serum level of $T_3$ was markedly increased: 0·31 and $> 50$ $\mu$g/l in hypothyroid and $T_3$-treated hypothyroid rats respectively). This result is consistent with studies showing an increased $C_{max}$ of TR in severe hyperthyroidism (Hamada *et al.* 1979; Nakamura *et al.* 1979), but not with that of Bernal *et al.* (1978) who observed no significant change in $T_3$ binding capacity in $T_3$-treated thyroidectomized rats. Strait *et al.* (1990) reported that c-*erbA* $\beta$1 mRNA levels were not altered in the liver of rats rendered hyperthyroid ($T_3$, 2 mg/kg body weight, injected intraperitoneally every 48 h for 5 d).

### *Expression of retinoic acid receptors*

After 3 or 5 weeks administration of PTU the $C_{max}$ and mRNA of RAR were decreased. This was thought to be due, at least in part, to the change in vitamin A status of the hypothyroid animals. It has been shown, for example, that a vitamin A-deficient status reduces $C_{max}$ of RAR (Haq *et al.* 1991; Kato *et al.* 1992). Neither of these variables was significantly altered after feeding the hypothyroid rats on a vitamin A-enriched diet, and in a previous study (Audouin-Chevallier *et al.* 1993) we observed an increased amount of RAR mRNA and an increased $C_{max}$ of proteins in normal rats fed on a similar enriched diet.

Thus the stimulatory effect of vitamin A on the expression of RAR in the nuclei of rat liver was not observed in the hypothyroid animals. This could be due to either an extragenomic and/or a genomic mechanism. Hypothyroidism reduced levels of retinol and subsequently of cellular RA, and it is known that RA determines the level of RAR via an up-regulation mechanism (Haq *et al.* 1991; Kato *et al.* 1992). Unliganded receptors are present in hypothyroid rats as we found that the binding capacity of TR was unaffected while $T_3$ level was drastically reduced. It is known that unoccupied receptors bind to the responsive elements of target genes and suppress the expression of the genes (Lavin *et al.* 1988; Damm *et al.* 1989). It has recently been suggested that thyroid hormone aporeceptor represses $T_3$-inducible promoters and blocks activity of retinoic acid receptors (Brent *et al.* 1989; Lee *et al.* 1994).

Following $T_3$ administration during 2 weeks to the hypothyroid rats both the binding capacity of RAR and their corresponding mRNA were increased, although the $C_{max}$ did not reach the level found in control rats. In hypothyroid rats treated with $T_3$ and

supplemented with vitamin A, the $C_{max}$ of RAR and levels of their mRNA were higher than following $T_3$ administration alone, and were not significantly different from control values. In this condition all TR were assumed to be occupied and a new equilibrium between the liganded receptors may lead to an up-regulation of RAR by RA resulting from an adequate retinol metabolism. This observation may be of practical significance as a vitamin A-supplemented diet appeared to improve the effect of $T_3$ administration on the binding properties of RAR in hypothyroid animals.

*Expression of glucocorticoid nuclear receptors*

Whereas TR and RAR are mainly located in cell nuclei, GR are located in both cytosol (GRC) and nuclei ($GR_n$). Thus the levels of GR in nuclei result from two distinct processes: (1) biosynthesis of receptors whose number in cells (GRC and $GR_n$) is dependent on GR mRNA levels, (2) translocation of receptors from cytosol to nuclei. Since there was no alteration in GR mRNA content in the untreated hypothyroid rats and those treated with $T_3$ or a vitamin A-enriched diet or a combination of both, the changes in binding capacity of $GR_n$ were assumed to stem from modifications in the translocation of GR from cytosol to nucleus. In fact, nuclear $GR_n$ results from a dynamic process, as the receptor is translocated from cytosol to nucleus and constantly diffuses out of the nucleus (Guiochon-Mantel & Milgron, 1993), although the mechanism of receptor translocation across the nuclear envelope and the involvement of putative regulatory factors remain to be elucidated. This knowledge will be crucial since, of course, it is the GR located in nucleus (i.e. $GR_n$) which are involved in the regulation of gene expression. It is known that the nuclear localization of $GR_n$ is under control of glucocorticoid hormone (Höck *et al.* 1989) and that $GR_n$ bear nuclear localization signals (Muller & Renkawitz, 1991; Ylikomi *et al.* 1992). Moreover, GR is a phosphoprotein (Aurichio, 1989) which is hyperphosphorylated after binding ligand (Orti *et al.* 1992; Bodwell *et al.* 1995). Phosphorylation of the receptor might influence its nuclear translocation (Orti *et al.* 1989), and certain kinases such as protein kinase C (PKC) have been suspected to catalyse this phosphorylation (Kido *et al.* 1987; Moudgil, 1990). This would suggest that any treatment inducing a significant change in thyroid status will affect kinase activity and in turn GR translocation. Fugassa *et al.* (1976) and Coleoni & De Groot (1980) showed that administration of $T_3$ to rats induced an increase in phosphorylation of the liver nuclear protein. Our results support this idea since we found a decreased $C_{max}$ of $GR_n$ in hypothyroid rats, which was enhanced by administration of $T_3$. The vitamin A-enriched diet was without effect on GR translocation in the liver of hypothyroid rats as the diet was probably not able to counteract the hypovitaminosis induced by PTU in these animals. In normal rats, the administration of RA has been found to enhance PKC activity in liver (Pailler-Rodde *et al.* 1991*a*) and the translocation of GR from the cytosolic to the nuclear compartment in hepatocytes (Audouin-Chevallier *et al.* 1995).

In conclusion, we showed that hypothyroidism affected the expression of nuclear hormone receptors in different ways. The decreased $C_{max}$ of RAR and TR were attributed mainly to reduced gene transcription, while the decreased $C_{max}$ of $GR_n$ was assumed to be due to a post-transcriptional event ascribed to reduced translocation of receptors from cytosol to nucleus. The mechanisms responsible for alterations in the level of transcription of RAR and TR genes, observed in hypothyroidism and in hypothyroid rats treated with $T_3$, are as yet unclear. However, it is known that any cellular event that affects the equilibrium between a receptor and its ligands or between the various receptors of the superfamily has profound effects on gene transcription. We suggest that the differing effects of administration of RA and a vitamin A-rich diet in PTU-induced hypothyroid rats

stemmed from the influence of reduced thyroid hormone levels on the bioavailability of vitamin A (retinol) and/or its metabolism into RA.

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