TRIIODOTHYRONINE BINDING PROTEINS IN RAT LIVER CYTOSOL

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SUMMARY. Triiodothyronine specifically binds to a liver cytoplasmic protein. This protein is distinct from T_3 nuclear receptor and from serum TBG, as shown by kinetic, sucrose gradient and electrophoretic studies. The cytosol-T_3 complex does not bind to DNA under conditions when NHP-T_3 does. The binding of T_3 to cytosol proteins shows a sigmoid relationship with the hormone concentration ; this sigmoid relationship is abolished by keeping the cytosol in cold. The sigmoid saturation curve and the rapid dissociation of the T_3-protein complex suggest a regulatory role of this protein in the supply of the hormone in the cell.

It is usually admitted that part of the biological role of thyroid hormones results from a primary effect on the control of gene expression (1). The accumulation of labelled T_3 in the nuclei of rat liver and kidney has been reported (2-7). In a previous work, using *in vitro* incubations of thyroid hormones with liver NHP, we demonstrated the existence of limited capacity, high affinity binding sites for L-T_3 (8), which is consistent with the role presently attributed to NHP in the control of cell growth and differentiation. Several laboratories have described iodothyronine binding proteins in cytosol from various tissues (9-13). In this work, we describe a specific cytoplasmic T_3 binding protein, different from the nuclear receptor, which shows a sigmoid saturation curve with T_3 concentration, suggesting a regulatory role of this protein in the supply of the hormone.

MATERIAL AND METHODS. [^{125}I] L-T_3 (CEA, Saclay, France : specific activity : 9-80 Ci/mM) was purified by Dowex 1 x 2 column chromato-

ABBREVIATIONS USED : NHP = chromatin non histone proteins ; T_4 = L-thyroxine ; T_3 = L-3,5,3' triiodothyronine ; T_2 = L-3,5 diiodothyronine ; Triac = 3,5,3' triiodothyroacetic acid ; MIT = L-monoiodothyronine ; TBG = serum thyroxine binding globulin.

graphy (14). L-T_3 (sodium salt), L-T_4 (free form), L-T_2 (free form) and Triac (diethanolamine salt) were from Sigma. Male euthyroid Wistar rats weighing about 150 g were used. Cytosol was prepared from extensively perfused livers, homogenized in 50 mM Tris HCl pH 7.55, 25 mM KCl, 0.25 M sucrose. NHP were extracted from liver nuclei as previously described (15). The incubation medium contained 2 mg cytosol proteins or 300 µg NHP in 50 mM Tris-HCl (pH 8.0), 1 mM Na EDTA, 1 mM β-mercaptoethanol to a final volume of 0.5 ml. Free and protein-bound hormones were separated by the charcoal method : 0.5 ml of a charcoal suspension (5 mg/ml Norit A in the incubation buffer) was added to each incubation sample, mixed immediately and kept at 0° for 15 min ; charcoal was then removed by centrifugation at 1,500 g for 5 min. In all these experiments the non specific binding measured in the presence of a 500-fold excess of cold T_3 never exceed 15 % of the total binding. Free and cytosol-bound radioactive T_3 present in the incubation medium, when analyzed by paper chromatography in butanol, ammoniac, water (72-250-178), behaved as T_3. Polyacrylamide gel electrophoreses were performed according to Sherman *et al.* (16) on 6 % acrylamide gels. Rat liver DNA was prepared by the method of Marmur (17). The binding of protein-hormone complexes to DNA was performed by incubation for 90 min at 0° and the samples were chromatographed on a Biogel A 5 m column (1.5 x 40 cm) at a flow rate of 60 ml per hr, 1 ml fractions were collected. [^{125}I] radioactivity was measured in a Packard Autogamma Spectrometer with an efficiency of 50 %. Protein concentration was estimated by the Lowry method (18), and DNA by the diphenylamine method (19).

RESULTS AND DISCUSSION.

Characteristics of T_3 binding to cytosol proteins : Association of T_3 with liver cytosol proteins was instantaneous at 21° ; at 0° the kinetics of the binding were much slower, the association was completed within 2 hrs and remained constant for at least 24 hrs (Fig. 1 A) ; the non specific binding was independent of the time and constant throughout the experiment. The kinetics of dissociation of the binding protein-T_3 complex were investigated with the use of charcoal incubated for various times with the complex. First order kinetics, with a t 1/2 of 20 min, were obtained at 0° (Fig. 1 B). When the dissociation was studied in the presence of an excess of unlabelled T_3, identical results were obtained. After 90 min, about 95 % of the specific binding complexes were dissociated. The kinetics of dissociation of the complexes were identical when proteins had been incubated in the presence of a high (2.10^{-8} M) or a low (1.10^{-9} M) concentration of the hormone. At 21° the disso-

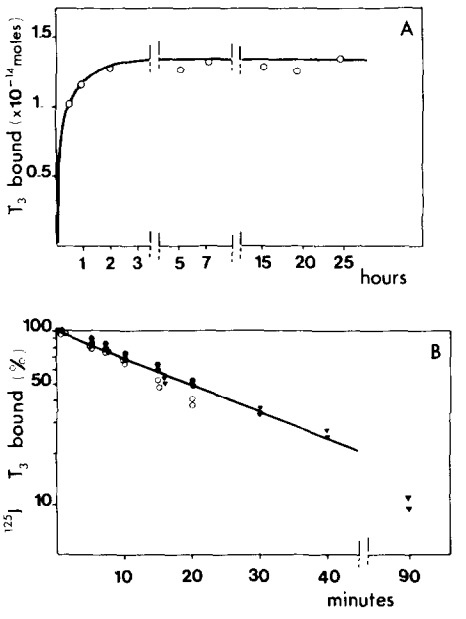

Figure 1. Time course of binding to liver cytosol proteins : A. Kinetics of association : cytosol proteins (2 mg) were incubated with 8.10^{-10} M labelled T_3 at 0° for various times. The bound radioactivity was determined after charcoal treatment. Each value was corrected for non specific binding by means of simultaneous experiments run in the presence of an excess of cold L-T_3. B. Kinetics of dissociation : cytosol proteins and labelled T_3 were incubated at 0° for 2 hrs. Charcoal suspension was then added (2.5 mg/ml). At various time intervals, 1 ml aliquots were centrifuged and the radioactivity of the supernatant measured ; 100 % represents the specific binding activity calculated after extrapolation at zero time. Experiments were performed with fresh cytosol (o) and cytosol kept in cold (o) in the presence of 10^{-9} or 2.10^{-8} M labelled T_3. Dissociation was also studied after addition of 2.5×10^{-6} M unlabelled T_3 (▽). At various times, the residual bound radioactivity was determined by the charcoal method (15 min at 0°) on 0.5 ml aliquots. The specific binding before addition of unlabelled hormone was takem as 100 %.

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ciation of the binding protein-T_3 complex also follows first order kineticswith a t 1/2 of 120 sec (*not shown*).

The relative affinity of the cytosol binding proteins towards iodothyronine was investigated by the competition technique using 2.10^{-8} M $\left[^{125}I\right]$ T_3 and 10^{-7} M non labelled competitors. The ^{125}I T_3 binding to cytosol was much more inhibited by T_3 than by T_4 and other analogues (Table I). Since the relative affinity for iodothyronines exhibited by cytoplasmic sites differs sharply from

Table I

L-T_3	100 %
L-T_4	35 %
L-T_2	65 %
Triac	56 %
MIT	0 %
Tyrosine	0 %

Table I. Specificity of T_3 binding : Cytosol proteins (2 mg) and radioactive T_3 ($2 \cdot 10^{-8}$ M) were incubated at 0° for 120 min., alone or in the presence of $2 \cdot 10^{-7}$ M of the indicated non labelled competitors. The bound radioactivity was determined by the charcoal method. 100 p.100 represents the competition in the presence of non labelled L-T_3.

the affinity exhibited by serum proteins (20), a contamination of cytosol by serum can be excluded.

After a 2 hr incubation at 0° of cytosol in the presence of 2 mM parahydroxymercuribenzoate, the binding capacity towards T_3 was completely abolished. This effect was reversed to 90 % by subsequent incubation (30 min) with 10 mM β-mercaptoethanol (*data not shown*).

Discrimination between nuclear and cytoplasmic sites : Kinetic and specificity studies suggest differences between nuclear and cytoplasmic binding proteins. It was confirmed by the following experiments :

- On a 5-20 % sucrose gradient, cytosol-T_3 complexes sedimented as a single narrow peak (Fig. 2) with a sedimentation constant close to that of serum transcortin (3.6 S)(21). This peak was abolished by more than 80 % when unlabelled T_3 was added to the incubation medium. The NHP-T_3 complexes have a lower sedimentation constant. In the same conditions, the TBG-T_3 complexes sediment slightly faster that cytosol complexes ;

- On 6 % polyacrylamide gels, the cytosol complex migrat-

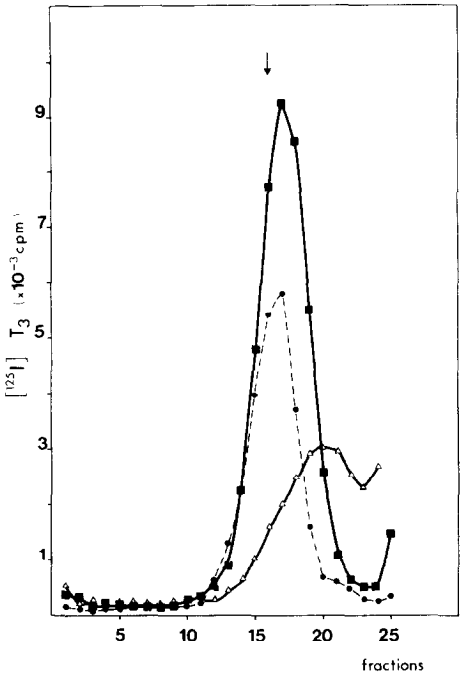

Figure 2. Sucrose gradient pattern of cytoplasmic and nuclear T_3 binding proteins. Cytosol proteins (7 mg) and radioactive T_3 (10^{-11} moles) were incubated in a final volume of 0.50 ml at 0° for 120 min. (■——■). A 0.2 ml aliquot was applied on a 5-20 % linear sucrose gradient prepared in incubation buffer ; centrifugation was performed at 35,000 rpm for 18 hrs at 4°, 210 µl fractions were collected and the radioactivity determined. The same experiments were performed with NHP (2.1 mg) (Δ——Δ) and serum proteins (7.5 mg) (●——●).

In all these experiments serum transcortin bound to tritiated corticosterone was used as a 3.6 S marker (arrow).

ed as an homogenous peak with a relative electrophoretic mobility (R_F) of 0.27 (Fig. 3) ; the R_F of the nuclear receptor was 0.35 ; an excess of cold T_3 completely abolished these peaks. In the same conditions the R_F of TBG was 0.69 ;

- one of the most important functions of a nuclear hormone receptor is its ability to bind to DNA in order to regulate gene expression. Fig. 4 shows that NHP-T_3 complexes, in the presence of DNA, eluted in the void volume of a Biogel A 5 column with DNA, whereas in the absence of DNA they eluted later ; these findings are in agreement with results of MacLeod and Baxter (22) obtained

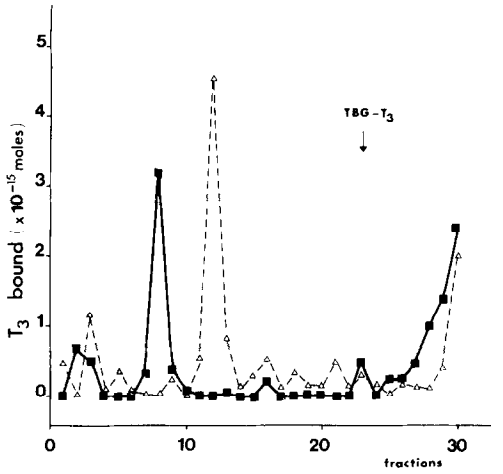

Figure 3. Polyacrylamide gel electrophoresis of T_3 binding proteins. Cytosol (2 mg) and 10^{-13} moles of labelled L-T_3 (■—■) were incubated at 0° for 2 hrs in a final volume of 0.4 ml ; simultaneous experiments were performed in the presence of an excess of non labelled T_3. Aliquots (0.2 ml) were loaded on 6 % polyacrylamide gels and subjected to electrophoresis for 120 min. at 4° with a current of 2 mA per gel. The gels were sliced and the radioactivity of each slice measured. The R_F were obtained by comparison with the migration of bromophenol blue. NHP (1.8 mg) (Δ---Δ) and serum proteins (2 mg) were incubated in the same conditions for 20 hrs at 0°. In all the experiments the non specific binding was substracted.

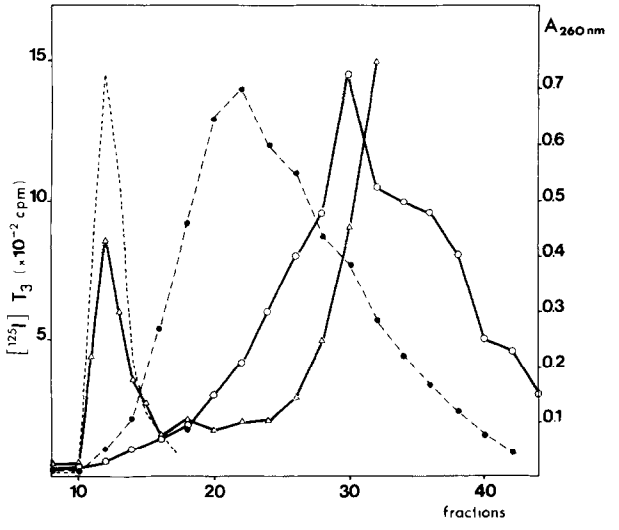

Figure 4. Binding to DNA of protein-T_3 complexes. Liver NHP (Δ—Δ) (3 mg), cytosol (o——o) (4 mg) or rat serum (•---•) (5 mg) were incubated with labelled T_3 ($2 \cdot 10^{-8}$ M) until the equilibrium was reached (24 hrs for NHP, 2 hrs for cytosol and serum) and then incubated with 150 µg of liver DNA for 90 min. at 0°. The samples were then chromatographed on a Biogel A5 column as described in the Methods section. The absorbance at 260 nm (----) was monitored by a Beckman DB spectrophotometer and the radioactivity of each fraction determined.

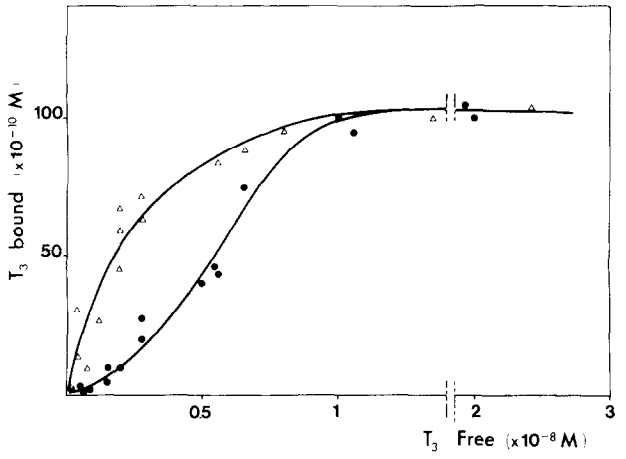

Figure 5. Saturation curve. Freshly prepared cytosol (●——●) and cytosol kept for 16 hrs at 0° (Δ---Δ) were incubated with increasing amounts of radioactive T_3 with or without a 500-fold excess of unlabelled T_3. The incubations were performed for 2 hrs at 0° with 2 mg proteins in 0.5 ml. The free hormone was adsorbed on charcoal and corrections were made for non specific binding.

after nuclei incubation. In the same conditions, no specific binding of T_3-cytosol complex to DNA was detected in the presence or in the absence of NHP, even after preincubation at 20° for 30 min.

Effect of T_3 concentration on the binding to cytosol sites : A sigmoid dependence on T_3 concentration was observed when cytosol proteins were incubated at 0° with increasing amounts of the hormone (Fig. 5). Identical results were constantly found in multiple independent experiments. In all cases, the non specific binding was linear and corrections were made for determination of the specific binding. When the experiments were performed with cytosol previously kept at 0° or at -20° for 16 hrs, the saturation of the proteins showed no more a sigmoid relationship with T_3 concentration. Pretreatment of cytosol with charcoal does not modify the binding properties of the proteins towards T_3. It is unlikely that keeping the cytosol in cold leads to an aggregation or to a dissociation of the binding proteins since their sedimentation constant in sucrose gradient was not modified (*not shown*).

The kinetics of dissociation, measured by the charcoal technique, were not significantly changed when cytosol kept in cold was used as a source of binding proteins instead of fresh cytosol (Fig. 1 B). The binding proteins were saturated in both cases (Fig. 5) with 2.10^{-8} M T_3 and the specific activity measured from the plateau was 24. 10^{-13} moles T_3 bound per mg proteins. With native cytosol, half saturation was obtained with 10.4×10^{-9} M T_3 ; it was 6.8×10^{-9} M T_3 with cytosol previously kept on cold.

In a previous work (8) we described a T_3 receptor in liver nuclei from euthyroid rats. On their way to the nucleus, thyroid hormones T_3 and T_4 traverse the cytoplasm where they bind to a variety of proteins (9-13). The question arises whether such a binding was required for the nuclear localization of the hormone. In this paper, we describe the properties of a cytosol binding protein for triiodothyronine which binds T_3 more avidly than T_4. It is a unique class of protein as shown by electrophoretic pattern and kinetics of dissociation. The capacity of cytosol for T_3 is 100 times higher than that of nuclei. This protein differs from nuclear receptors by its kinetic properties, sedimentation constant and electrophoretic mobility. Moreover it does not bind to DNA under conditions when the nuclear receptors do so ; either we have not found the conditions for DNA binding of the cytoplasmic complex, or the cytosol proteins might not be directly related to the nuclear receptors. In agreement with this last hypothesis, nuclear binding sites have been demonstrated in the absence of cytosol proteins (23) or of the hormone (6).

Moreover nuclear receptors tightly bind to DNA in the absence of the hormone (*data not shown*). It is then likely that T_3 penetrates into the nucleus in a free form and modifies the activity of a

receptor present already in chromatin. The sigmoid shape of the saturation curve of the cytosol binding proteins favors the hypothesis that these proteins contain more than one binding site per molecule with a cooperative effect between the different sites. In calf uterus, the interaction between the estradiol binding proteins and the hormone could not be described either by simple reaction schemes (24). Our results suggest a regulatory role of the cytosol proteins in the retention and the supply of the hormone to the various cell components including mitochondria and nucleus.

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