IN VITRO TRIIODOTHYRONINE BINDING TO NON-HISTONE PROTEINS FROM RAT LIVER NUCLEI.

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SUMMARY. In vitro incubations of non-histone proteins from rat liver nuclei with labelled L-3, 5, 3' triiodothyronine demonstrated the existence of high affinity, limited capacity binding sites for the hormone in this protein group; the affinity was found identical for triiodothyroacetic acid and lower for L-thyroxine. Binding ability was highly temperature dependent. At 4°C, the rate constant of association was 0.9×10^7 M^{-1} h^{-1} and the rate constant of dissociation was 0.015 h^{-1}. The dissociation constant K_d was calculated from these data or measured by Scatchard analysis and found to be between 1.6 and 5×10^{-9} M. The maximum binding capacity was 10^{-13} moles of L-3, 5, 3' triiodothyronine per 100 µg non-histone proteins or 6000 hormone molecules per nucleus. Protein binding had a half-life of 20 hours at 4°C, in the absence of hormone, but was found to be very stable in the presence of hormone.

Investigations using in vivo injections of labelled thyroid hormones have shown the presence of limited capacity binding sites for L-T_3 in the nuclei of rat liver and kidney (1-5). Similar results were found by incubating labelled hormones with intact pituitary cells or with nuclei (6-8). In this paper, we provide evidence to show that L-T_3 binds in vitro to NHP extracted from rat liver nuclei. The evidence is supported by kinetic, affinity, specificity and stability experiments.

MATERIALS AND METHODS. [^{125}I] L-T_3 (C.E.A., Saclay, France, specific activity 65 to 160 Ci per mM) was purified by Dowex 1 x 2 column chromatography (9) and contained less than 1% iodide. L-T_3 (sodium salt), L-T_4 (sodium salt) were obtained from Calbiochem, L-T_2 (free form), triac (diethanolamine salt) were from Sigma. All others chemicals were analytical grade. Male Albino Wistar rats weighing about 150 g were used. NHP were extracted from liver nuclei and partially purified as previously described (10). The incubation medium contained 10 mM Tris-HCl,(pH 7.5)0.4 M NaCl, 1 mM Na EDTA, 1 mM β mercapto-ethanol, 0.1 % sodium azide; Na EDTA and β mercaptoethanol were included in

ABBREVIATIONS USED. NHP, non-histone nuclear proteins; L-T_4, L-thyroxine; L-T_3, L-3, 5, 3'triiodothyronine; L-T_2, L-3, 5 diiodothyronine; triac, 3,5, 3'triiodothyroacetic acid; MIT, L-monoiodotyrosine; DIT,L-diiodotyrosine.

order to inhibit the formation of covalent complexes between L-T_3 and proteins (11). The final incubation volume was 0.46 ml and contained 300 to 350 µg of NHP. Free and protein-bound hormone were separated by two methods. a/. The incubation medium was usually chromatographed on Biogel P-10 (Bio-Rad, Richmond, Calif) columns (14 x 1.2 cm) equilibrated and eluted with 50 mM Tris-HCl,(pH 7.5)1 mM Na EDTA, 1 mM β mercaptoethanol, 0.1% sodium azide. The bound $\left[^{125}I\right]$ L-T_3 was eluted in the void volume and the free $\left[^{125}I\right]$ L-T_3 was eluted with three and a half times the total volume ; recovery of radioactivity was 100%. b/. In rapid association binding studies, free hormone was separated by a charcoal-dextran method. The suspension was made of Norit A (Nutritional Biochemicals Corp., Clev., Ohio) 300 mg/100 ml, and Dextran T-20 (Pharmacia, Uppsala, Sweden) 30 mg/100 ml in the incubation medium buffer and kept at 0° C. One ml of this suspension was transferred to the incubation tubes, mixed immediately and kept, without further mixing, at 0° C for 15 minutes (preliminary experiments showed no significant dissociation of the bound hormone by incubation with charcoal under these conditions, between 5 and 30 minutes). Charcoal was then removed by centrifugation at 1500 g for 15 minutes at 4° C. Control incubations without NHP were simultaneously performed for "blank" corrections. The "blank" values of the supernatants were always found to be less than 1% of the total radioactivity. "Non specific" binding was estimated as described in the legend to Fig. 1. "Specific" binding was calculated by substracting the above value from the total. NHP bound radioactivity at equilibrium was quantitatively extracted by methanol-NH_4OH (99 : 1) and chromatographed on thin-layer cellulose (12) ; 90% of the radioactivity remained in the form of L-T_3. ^{125}I was measured in a Packard Autogamma Spectrometer with an efficiency of 45% and with a counting accuracy of 1%. Protein concentration was estimated by the Lowry et al (13) method.

RESULTS. Association of $\left[^{125}I\right]$ L-T_3 with NHP was highly temperature dependent. At 4° C,it was very slow and reached a maximal value after 24 hours (Fig. 1A),

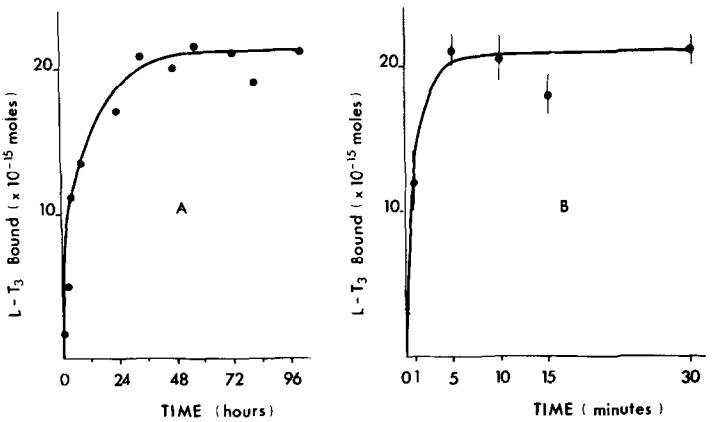

Figure 1. Time course of $[^{125}I]$ L-T_3 binding to NHP. A/ At 4° C : NHP (320 µg) and $[^{125}I]$ L-T_3 (3.5×10^{-13} moles) were contained in a final volume of 0.46 ml per incubation and were chromatographed at the indicated time intervals, on Biogel P - 10 columns, as described in the Methods. The bound $[^{125}I]$ L-T_3 fraction was collected. Each value was corrected for "non-specific" binding by means of simultaneous incubations run in the presence of excess cold L-T_3 (1.7×10^{-10} moles). B/ At 22° C : incubations were performed at the same concentrations as above ; at the indicated time intervals, the association was stopped by adding the charcoal-dextran suspension as described in the Methods ; "non-specific" binding correction was as in A ; bars indicate ± SD of triplicates.

while at 22° C, the association was complete within 5 minutes (Fig. 1B). On the other hand, "non-specific" binding was independent of time, whether at 4° C or at 22° C and remained constant throughout the experiments (data not shown). The rate of dissociation was investigated, at 4° C, by adding a large excess of cold L-T_3, after the association had reached equilibrium (24 hours at 4° C) (Fig. 2). The dissociation process kinetics were first-order, with a half-dissociation time of 52 hours. In control experiments, where dissociation was obtained by adsorption of unbound $[^{125}I]$ L-T_3 on charcoal, similar results were observed. The kinetic rate constants of the L-T_3-NHP binding reaction were calculated from these data. The rate constant of association (k_{+1}) at 4° C was 0.9×10^7 $M^{-1}.h^{-1}$ and the rate constant of dissociation (k_{-1}) at 4° C, was 0.015 h^{-1}. From the k_{-1}/k_{+1} ratio the dissociation constant K_d of NHP for L-T_3 was calculated and found to be 1.6×10^{-9}M. The K_d was also experimentally determined at equilibrium (24 hours at 4° C) and the data were plotted according to Scatchard (14) (Fig. 3). The apparent dissociation

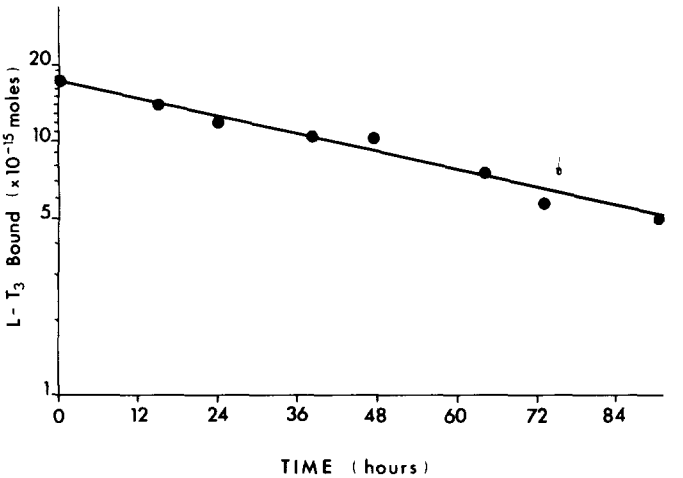

Figure 2. Time course of dissociation of $[^{125}I]$ L-T_3 bound to NHP, at 4° C. NHP (300 μg) and $[^{125}I]$ L-T_3 (3.5×10^{-13} moles) in a final volume of 0.46 ml were incubated at 4° C for 24 hours. Then an excess of cold L-T3 (1.7×10^{-10} moles) was added. The radioactivity bound to NHP was isolated, at the indicated time intervals, by Biogel P - 10 column chromatography (see Methods). "Non-specific" binding correction was as described in the legend to Fig. 1A.

constant K_d was 5.10^{-9}M. However, "non-specific" binding was not corrected and the above value was probably over-estimated. Therefore, calculated and experimentally measured K_d values were found to be in good agreement. Furthermore, the maximal binding capacity of NHP for L-T_3 estimated from the Scatchard plot was 10^{-13} moles L-T_3 bound per 100 μg NHP. This represents 6000 molecules of L-T_3 bound per liver nucleus at saturation, since 100 μg NHP are extracted from 10^7 nuclei, in our method. The specificity of the NHP binding ability was investigated using a competition technique, described in the legend to Table 1. The $[^{125}I]$ L-T_3 binding to NHP was equally inhibited by non-labelled L-T_3 and triac and to a smaller extent by L-T_4. L-T_2 was even less effective, yet a significant inhibition could be demonstrated. MIT and DIT were totally inactive. The stability of the NHP-hormone complex in the association experiments was remarkable, since it remained unaltered for up to three days (Fig. 1A). This was therefore, reexamined when NHP were kept at the same conditions, but in the absence of hormone. It was found that they gradually lost their ability to bind specifically L-T_3, with an apparent half-

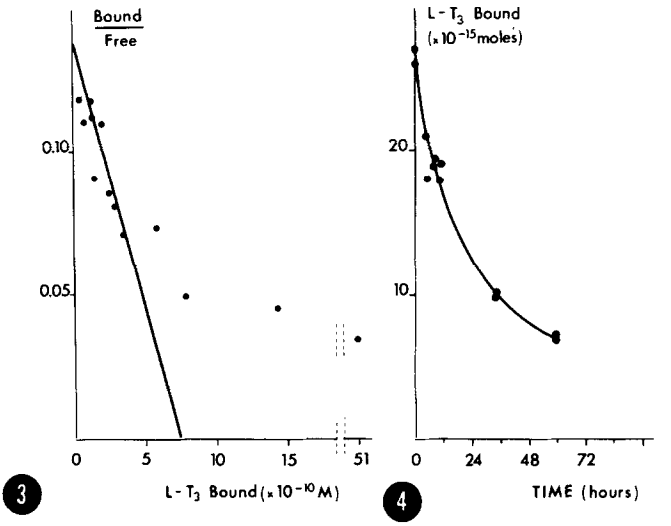

Figure 3 (left). Scatchard analysis of L-T_3 bound to NHP. NHP (320 µg) and [^{125}I] L-T_3 (3.5 x 10^{-13} moles) in a final volume of 0.46 ml were incubated in the presence of various amounts of cold L-T_3, at 4° C for 24 hours. Then, the protein bound radioactivity was isolated by Biogel P - 10 column chromatography (see Methods). Results are plotted as the ratios of bound to free hormone versus bound hormone concentrations.

Figure 4 (right). NHP stability. NHP solution kept at 4° C, was tested for its L-T_3 binding ability, at the indicated time intervals, by incubation with [^{125}I] L-T_3 at 4° C for 24 hours. The concentrations and the "non-specific" binding corrections were as described in the legend to Fig. 1A. The separation of free from protein bound radioactivity was performed by the charcoal-dextran technique (see Methods).

degradation time of 20 hours (Fig. 4). These data indicate that the presence of L-T_3 protected the NHP binding sites from degradation. Specific binding was partially abolished by incubation with sulfhydryl reagents, sodium parahydroxymercuribenzoate or N-ethyl maleimide. The effect of sodium parahydroxymercuribenzoate was found to be reversible by subsequent incubation with β mercapto ethanol (table II).

DISCUSSION. Previous studies demonstrated the existence of nuclear binding sites for L-T_3 and L-T_4, by *in vivo* injection techniques (1-5) or by incubation with intact cells or with isolated nuclei (6-8). Using *in vitro* incubation of thyroid hormones with partially purified liver nuclear non-histone proteins, we have demonstrated the existence of limited capacity, high affinity binding sites for L-T_3, within this protein group. It should be

L-T_3	100 %	± 4
Triac	102 %	± 5
L-T_4	60 %	± 3
L-T_2	40 %	± 4
DIT	0 %	
MIT	0 %	

Table I. Specificity experiments. NHP (300 µg) and [^{125}I] L-T_3 (3.5×10^{-13} moles) in a final volume of 0.46 ml were incubated at 4° C for 24 hours, alone or in the presence of 10^{-10} moles of the indicated non-labelled compounds. The bound radioactivity was isolated by Biogel P - 10 column chromatography (see Methods). 100 % represent the inhibition of [^{125}I] L-T_3 binding caused by the presence of non-labelled L-T3. Each figure represents the mean of three determinations ± SD.

Control	100 %	± 5
N-ethylmaleimide (10^{-3} M)	25 %	± 3
Sodium parahydroxymercuribenzoate (2×10^{-4} M)	44 %	± 5
Sodium parahydroxymercuribenzoate (2×10^{-4} M) + mercaptoethanol (2×10^{-3} M)	84 %	± 6

Table II. Role of sulfhydryl groups. NHP were incubated for 2 hours at 4° C, alone or in the presence of the indicated compounds ; β mercaptoethanol was added, where indicated, for an additionnal 2 hours incubation. The binding ability was tested in the presence of [^{125}I] L-T_3 at 22° C for 15 minutes, using the charcoal technique for bound radioactivity separation (see Methods). The NHP and [^{125}I] L-T_3 concentrations and the "non-specific" binding corrections were as in the legend to Fig. 1A ; the NHP preparation did not contain β mercaptoethanol. Results are expressed as the percentage of binding to nontreated NHP ; each figure represents the mean of three determinations ± SD.

noted that our kinetic studies disclosed that the binding is highly temperature dependent and that the rate of association is very slow at 4° C. One of the reasons, therefore, why the in vitro binding of L-T_3 to NHP could not, as

yet, be demonstrated may possibly have been the use of short incubation times at 4° C (5). The total number of L-T_3 binding sites calculated from our data (6000 hormone molecules per nucleus) is in good agreement with other authors' results (7800 hormone molecules per nucleus) using different experimental conditions (8). The K_d values obtained from two different methods were found to be in good agreement. When our results are compared with those obtained by other methods, a gradual increase of the values is observed : by in vivo injection techniques (2), the K_d obtained was 2.1×10^{-12} M, by incubation with intact cultured cells methods, it was 2.9×10^{-11} M (7) and with isolated nuclei, it was 1.65 to 2.1×10^{-10} M (8). Our binding studies with partially purified NHP, showed a K_d of 1.6 to 5×10^{-9} M. The decrease of apparent affinity observed at increasing isolation and purification levels is a frequent finding in biochemical processes. Moreover, we demonstrated a stabilization of the binding ability, in vitro, by the presence of the ligand. In the specificity experiments, NHP showed equal affinity for L-T_3 and triac and less affinity for L-T_4. This clearly discriminates them from plasma binding proteins, which bind L-T_4 much stronger than L-T_3, whether in man (15) or rat (16). Also, our specificity data are similar to the findings obtained by in vivo injection methods (17).

The pathways of thyroid hormone penetration in the cell, particularly the existence of a cytoplasmic receptor and its relationship with the nucleus, as well as their putative action on gene activity, are not completely established. The demonstration of high affinity binding sites for L-T_3 in NHP supports the hypothesis that this hormone plays a role in genome transcription, since NHP have been shown to selectively bind to DNA and to be involved in the control of RNA synthesis (18). Therefore, studies on the NHP-hormone complex may be a useful tool for the experimental approach to these problems.

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