A new unextracted-sample radioimmunoassay method for hepatic endogenous nuclear L-tri-iodothyronine content

Validity of its use in determining nuclear receptor binding characteristics

Toshihiro YAGURA and Paul G. WALFISH*

Thyroid Research Laboratory, Harold Tanenbaum Department of Research, and Department of Medicine, Endocrine Division, Mount Sinai Hospital, University of Toronto Medical School, Toronto, Ont. M5G 1X5, Canada

(Received 27 May 1982/Accepted 24 August 1982)

Endogenous L-tri-iodothyronine content in an hepatic nuclear extract was measured by a new unextracted-sample radioimmunoassay method using 8-anilino-naphthalene-1-sulphonic acid to inhibit the L-[¹²⁵I]tri-iodothyronine binding to the nuclear L-tri-iodothyronine receptor within the extract. For this method, the lower sensitivity limit was 3.125 pg/tube, the recovery of added L-tri-iodothyronine was 90–120%, and the between-assay coefficient of variation was 10%. The amount of endogenous L-tri-iodothyronine was 10–40 pg/0.2 ml of hepatic nuclear extract from euthyroid rats, compared with less than 3.125 pg/0.2 ml from thyroidectomized rats. The results obtained by this new method were compared with a Sephadex G-25 column extracted-sample radioimmunoassay method and showed a good agreement. The values for the endogenous L-tri-iodothyronine content were utilized to correct for the L-tri-iodothyronine concentration within the binding assay mixture in order to accurately determine by Scatchard analysis the binding characteristics of the nuclear L-tri-iodothyronine receptor. The validity of the correction for endogenous L-tri-iodothyronine was demonstrated by using a nuclear extract from a thyroidectomized rat which was preincubated with a small known amount of L-tri-iodothyronine before determining the nuclear L-tri-iodothyronine receptor binding characteristics. When the Scatchard plots were corrected for the preincubated dose, the results obtained were similar to true values, but they were falsely lower when not corrected. It is concluded that the necessity and validity of using endogenous L-tri-iodothyronine corrections in the Scatchard analytical computations of the nuclear L-tri-iodothyronine receptor binding characteristics has been demonstrated, being particularly more important for affinity constant than maximum binding capacity.

Initial methodology to characterize the nuclear T₃ receptor and study the effects of various physiological and pathological alternatives were per-

Abbreviations used: T₃, L-tri-iodothyronine; K_a, affinity constant; C_{max}, maximum binding capacity; ANS, 8-anilino-naphthalene-1-sulphonic acid sodium salt; TGKM buffer, 50 mM-Tris/glycine buffer (pH 8.5)/0.4 M-KCl/5 mM-MgCl₂; BSA-TG buffer, 0.03% bovine serum albumin/50 mM-Tris/glycine buffer, pH 8.5.

* To whom request for reprints should be directed at the following address: Thyroid Research Laboratory and Endocrine Division, Mount Sinai Hospital, Suite no. 639–640, 600 University Avenue, Toronto, Ont. M5G 1X5, Canada.

formed by Oppenheimer *et al.* (1972, 1974a,b), who used 'in vivo' assay techniques. 'In vitro' assay methods for determining the receptor binding characteristics, K_a and C_{max}, were developed with purified whole nuclei by Samuels & Tsai (1973, 1974), Koerner *et al.* (1974) and DeGroot & Torresani (1975). Studies by Surks *et al.* (1973) and Samuels *et al.* (1974) demonstrated that the nuclear T₃ receptor was a chromatin non-histone acidic protein that could be extracted into a buffered 0.4 M-KCl solution. The binding characteristics of the receptor were readily assayed by Scatchard (1949) analysis, a small amount of such an extract being used (Thomopoulos *et al.*, 1974; Torresani & DeGroot, 1975). However, when a nuclear extract is

used as the receptor preparation in the receptor binding assay for Scatchard analysis, two precautions must be considered in the interpretation of the data. Firstly, endogenous nuclear T_3 is also extracted simultaneously with the receptor into the extract (Surks *et al.*, 1973; Samuels *et al.*, 1974) and should be incorporated into the Scatchard analysis, since Hamada *et al.* (1979) and Nakamura *et al.* (1979a,b) have shown that the binding characteristics of the receptor were lower whenever the endogenous nuclear T_3 was not considered in the calculation. Accordingly, the correct amount of T_3 in a binding assay mixture for Scatchard analysis of the receptor binding characteristics is the sum of the endogenous T_3 in the nuclear extract used as a receptor preparation as well as the added T_3 (labelled or unlabelled with radioactive tracer) employed to provide a concentration gradient. Secondly, it is important to ensure that the analysis of the receptor binding characteristics from Scatchard analysis be performed under conditions of equilibrium between the receptor, the endogenous T_3 and added T_3 . Silva *et al.* (1977) have demonstrated that incubation at 0°C does not achieve the desired conditions of equilibrium and showed that a pre-warming of the mixture was necessary to optimize the Scatchard analysis.

For the measurement of the endogenous T_3 contents in the nuclear extract, it is convenient to use a radioimmunoassay method. However, similar to the problems encountered for a direct radioimmunoassay of T_3 in serum, wherein the presence of thyroid-hormone-binding proteins (particularly thyroxine-binding globulin) must be eliminated to prevent its competition with added T_3 , antibody and T_3 tracer, the measurement of endogenous T_3 from a nuclear extract also requires the removal of competing nuclear T_3 -receptor binding sites. Nakamura *et al.* (1979a) used a small Sephadex G-25 column to separate the nuclear-receptor-binding sites from endogenous T_3 before utilizing a T_3 radioimmunoassay. Previous reports had established the efficacy of ANS in saturating thyroxine-binding globulin serum-protein binding sites and thereby permitting the direct radioimmunoassay measurement of serum thyroxine and T_3 by an unextracted-sample method (Chopra *et al.*, 1972). In the present study, we established a direct endogenous nuclear T_3 radioimmunoassay method by the addition of ANS to the assay mixture to saturate competing T_3 binding sites in the nuclear extract, which thereby avoided the need for prior column extraction. In addition, we re-evaluated the optimal assay conditions for the Scatchard analysis of the nuclear T_3 receptor binding characteristics in order to achieve equilibrium between the receptor, the endogenous T_3 and added T_3 tracer.

This work was presented in part on the Scientific

Program of the 64th Annual Meeting of The Endocrine Society in San Francisco, 16–19 June 1982 (abstr. no. 113), as well as in the Scientific Program and Proceedings of the 2nd Asia and Oceania Thyroid Association Meeting in Tokyo, Japan, 19–22 August 1982 (abstr. no. 128).

Materials and methods

Materials

T_3 , dithiothreitol and an anionic-exchange resin, Dowex-1 (Cl^- form; 200–400 dry mesh), were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Triton X-100 was obtained from Amersham Corp., Arlington Heights, IL, U.S.A. [^{125}I] T_3 , (sp. radioactivity 450–500 mCi/mg) was a product of Abbott Radiopharmaceuticals, North Chicago, IL, U.S.A.

Experimental animals

Normal male 11–17-month-old Long-Evans rats (500–700 g body wt.) were obtained from Canadian Breeding Laboratories, Quebec, Canada. When required, 10-month-old hypothyroid rats were prepared, 3 weeks before being used, by thyroidectomy under light diethyl ether anaesthesia, followed by a low-iodine test diet (ICN Pharmaceuticals, Cleveland, OH, U.S.A.) and a 1% calcium lactate supplement in their drinking water.

Preparation of purified hepatic nuclei

The purified hepatic nuclei were prepared by the method of Torresani & DeGroot (1975).

Preparation of nuclear extract

The purified nuclei obtained from 7 g of tissue were suspended in 7 ml of an extraction buffer (TGKM buffer). This suspension was stirred gently at 4°C for 60 min as described by Hamada *et al.* (1979). The mixture was centrifuged at 102 000 g for 45 min. The resultant supernatant, the nuclear extract, was enriched routinely with dithiothreitol to a final concentration of 1 mM.

Procedures for direct radioimmunoassay of endogenous T_3 in nuclear extracts

Experiments were performed to determine the optimum assay conditions for the direct radioimmunoassay of the endogenous T_3 in a nuclear extract (see the Results section). Specific anti- T_3 antiserum was raised in a rabbit by the method of Chopra *et al.* (1972). The assay mixture consisted of 0.2 ml of a nuclear extract as an endogenous T_3 sample or a standard T_3 solution containing 0–800 pg of T_3 in TGKM buffer, 0.1 ml of [^{125}I] T_3 (about 5000 c.p.m.) in BSA-TG buffer, 0.05 ml of 50 mM-dithiothreitol, 0.05 ml of 0.4% ANS and 0.1 ml of anti- T_3 antiserum, diluted 1:12 000 with 75 mM-barbital buffer, pH 8.6.

The mixture was incubated at 4°C for 24 h, then 1 ml of 10% (w/v) ice-cold resin slurry of Dowex-1 (Cl⁻ form; 200–400 dry mesh) in TGKM buffer was added. The free T₃ and [¹²⁵I]T₃ was adsorbed on the resin and was spun down to the bottom of the tube after centrifugation at 1000 g for 4 min at 4°C, whereas the bound T₃ fraction remained in the supernatant. Both the supernatant and the precipitate were counted for radioactivity to determine the apparent ratio of the bound form to the total [¹²⁵I]T₃ for the standards and unknown samples.

The non-specific binding ratio of [¹²⁵I]T₃ in the assay mixtures for the standard curve was determined by measuring an assay mixture not containing the antiserum or the nuclear extract. The non-specific radioactivity was subtracted to obtain the final B/T (bound radioactivity/total radioactivity) values for the standard curve.

The small residual amount of persistent binding of [¹²⁵I]T₃ to the nuclear T₃ receptor (considered to be non-specific in nature) was determined in the presence of 200 µg of added ANS/tube, a concentration that was found to minimize the receptor binding (see the Results Section). The non-specific binding for each nuclear extract sample was determined by analysing an assay mixture which contained the nuclear extract but not the antiserum and subtracted from the apparent bound radioactivity obtained when the antiserum was present. The resulting B/T values were then used in computing the endogenous T₃ receptor values.

Radioimmunoassay measurement of endogenous nuclear T₃ after separation from the nuclear T₃ receptor

When indicated, the endogenous nuclear T₃ in a nuclear extract was separated from the receptor by using a small Sephadex G-25 column and measured by the radioimmunoassay method as described by Nakamura *et al.* (1979a).

Measurement of protein and DNA

Protein was measured by the method of Hartree (1972) and DNA was determined by the method of Burton (1967) after extraction from liver by a modification (Schneider, 1957) of the original method (Schmidt & Thannhauser, 1945). (Actually the DNA is separately determined from a portion of the final nuclear suspension before the nuclei are further extracted using TGKM buffer for determining endogenous T₃ and nuclear-receptor-T₃ binding characteristics. Although the TGKM nuclear extracts are devoid of DNA, the amount of DNA determined in the nuclear suspension before extraction has been used to standardize the expression of endogenous T₃ and C_{max} results obtained per 0.2 ml of the final extract; e.g., If the final nuclear pellet has 630 µg of DNA and 7 ml of TGKM buffer is

used for extraction, then 0.1 ml of this final nuclear extract is considered to be equivalent to 9 µg of DNA. Accordingly, the final endogenous nuclear T₃ and C_{max} values obtained would be multiplied by 1000/9 in order to express the results as mg of DNA equivalent nuclear extract.)

Nuclear T₃ receptor binding assay for binding characteristics

The incubation mixture of the nuclear T₃ receptor binding assay for the binding characteristics by Scatchard analysis consisted of 0.1 ml of a nuclear extract, 0.05 ml of BSA-TG buffer containing 0.01–0.2 pmol of [¹²⁵I]T₃ and 0.05 ml of BSA-TG buffer. When a non-specific binding ratio was assayed, it contained 1000 pmol of unlabelled T₃ and 0.05 ml of 25 mM-dithiothreitol.

After prewarming at 30°C for 50 min, the mixtures were kept at 4°C for 19 h. Then the same separation procedure of the free and the bound forms as used in the endogenous nuclear T₃ radioimmunoassay was performed and the ratio of the bound to total radioactivity was calculated. The non-specific binding ratio was subtracted after the addition of an excess of unlabelled T₃ (1000 pmol/tube). The binding characteristics were calculated from these results by Scatchard (1949) analysis, which was performed either without or with a correction for the endogenous T₃ content present in the nuclear extract.

Statistical methods

When statistical analyses were performed, the results were analysed with a non-paired two-tail Student's *t* test.

Results

Endogenous nuclear T₃ direct radioimmunoassay method

Inhibition of binding of [¹²⁵I]T₃ to nuclear T₃ receptor by ANS. The effects of ANS on the binding of [¹²⁵I]T₃ to the nuclear T₃ receptor and/or the anti-T₃ antibody in the reaction mixture for the endogenous nuclear T₃ radioimmunoassay was studied (Fig. 1). As shown by curve NT₃R in Fig. 1, 56.5% of the [¹²⁵I]T₃ was bound to the receptor in the absence of ANS. However, in the presence of more than 200 µg of ANS, the binding of [¹²⁵I]T₃ to the receptor was minimal. On the other hand, the binding of [¹²⁵I]T₃ to the antibody was gradually reduced when the concentration of ANS was increased (Fig. 1, curve AB). Note that the extent of the decrease in the binding to the antibody was smaller than that of the receptor binding. When the mixture contained both the nuclear extract and the antibody (Fig. 1, curve NT₃R + AB), note that the binding of [¹²⁵I]T₃ without ANS was similar to that

Fig. 1. Effects of ANS on binding of $[^{125}\text{I}]\text{T}_3$ to nuclear T_3 receptor (NT_3R) and/or anti- T_3 antibody (AB) in endogenous nuclear T_3 direct radioimmunoassay mixture

The standard reaction mixture for a nuclear extract sample, which was described for the nuclear endogenous T_3 radioimmunoassay in the Materials and methods section, was prepared in the experiment represented by curve $\text{NT}_3\text{R} + \text{AB}$, except that the ANS concentration varied from 0 to $400\text{ }\mu\text{g}/\text{tube}$ as shown on the abscissa. The mixture was then incubated and the free and bound forms were separated according to the standard procedures. In the experiment represented by curve AB the nuclear extract was omitted from the assay mixture and replaced with TGKM buffer. In the experiment shown by curve NT_3R , the antiserum was omitted and replaced by the barbital buffer. The non-specific binding ratio of $[^{125}\text{I}]\text{T}_3$ in this experiment was measured at each concentration of ANS by assaying a mixture that contained the corresponding buffers instead of the nuclear extract or the antiserum. The ordinate shows the ratio of the bound form to the total $[^{125}\text{I}]\text{T}_3$ (B/T) after subtraction of the non-specific binding expressed as a percentage. All measurements were performed in triplicate and expressed as the mean $\pm$ s.e.m.. Note that the binding of $[^{125}\text{I}]\text{T}_3$ in the presence of the nuclear extract alone (curve NT_3R) was minimal when the amount of ANS/tube was $200\text{ }\mu\text{g}$ or greater. Also note that the binding of $[^{125}\text{I}]\text{T}_3$ to the antiserum in the presence of $200\text{ }\mu\text{g}$ of ANS was still sufficient to permit the radioimmunoassay of the endogenous T_3 in a nuclear extract (see curve AB).

which occurred when the antibody alone (curve AB) was present. In the presence of ANS, the binding in curve $\text{NT}_3\text{R} + \text{AB}$ was clearly less than in curve AB. Since the binding of the receptor to $[^{125}\text{I}]\text{T}_3$ is

Fig. 2. An example of a typical standard curve generated for the direct (unextracted-sample) radioimmunoassay of endogenous T_3 in nuclear extracts

The radioimmunoassay procedure was performed in duplicate as described in the Materials and methods section. B/B_0 (%) and T_3 content (pg/tube) were plotted on the ordinate and abscissa respectively in a logit/log scale to obtain a straight-line relationship. B_0 = the $[^{125}\text{I}]\text{T}_3$ radioactivity (c.p.m.) bound to T_3 antibody for the initial point of the standard curve, which is determined in the absence of added T_3 standard. B = the $[^{125}\text{I}]\text{T}_3$ radioactivity (c.p.m.) bound to T_3 antibody in the presence of either various known amounts of T_3 standards or nuclear extracts with various unknown amounts of endogenous nuclear T_3 . After subtraction for the contribution of non-specific $[^{125}\text{I}]\text{T}_3$ bound radioactivity (c.p.m.) (i.e., observed bound in the absence of T_3 antibody), which was separately determined for either standards or unknown nuclear extracts, the final B/B_0 values were computed as a percentage, based upon $\text{B}_0 = 100\%$, to directly determine from the standard curve the endogenous T_3 present in unknown nuclear-extract samples.

reduced to a minimum by an increase of ANS, it was considered that the binding of $[^{125}\text{I}]\text{T}_3$ would be primarily to the antibody and that any subsequent reduction in the $[^{125}\text{I}]\text{T}_3$ binding to the antibody could only be due to a competitive-displacement effect resulting from the endogenous T_3 contained within a nuclear extract.

A small amount of binding of $[^{125}\text{I}]\text{T}_3$ to the receptor remained even in the presence of an excess (more than $200\text{ }\mu\text{g}$ of ANS; Fig. 1, curve NT_3R). Subsequently the amount of $[^{125}\text{I}]\text{T}_3$ bound to the antibody in the presence of a nuclear extract and $200\text{ }\mu\text{g}$ of ANS/tube was determined as the difference between the radioactivity of the bound form obtained when the antibody and a nuclear extract

were both present (curve NT₃R + AB) compared with that amount of binding observed when only a nuclear extract was present in the absence of the antibody (curve NT₃R). Hence, for the standard procedure of the endogenous T₃ radioimmunoassay, 200 µg of ANS/tube was selected, since this concentration was observed to be sufficient for minimizing the binding of the receptor to [¹²⁵I]T₃, and at the same time did not significantly interfere with the binding of T₃ to the antibody. After 24 h of incubation at 4°C the binding of [¹²⁵I]T₃ was observed to reach equilibrium (results not shown).

Standard curve of endogenous T₃ radioimmunoassay in nuclear extract. A standard curve for endogenous nuclear T₃ radioimmunoassay is shown in Fig. 2. The percentage of total (T) [¹²⁵I]T₃ radioactivity added which was bound (B) to the T₃ antibody before adding unlabelled T₃ is defined as B₀. After subtraction of the non-specific-binding radioactivity B₀/T × 100 values ranged from 30 to 40%, and the lower limit of detection was usually 3.125 pg of T₃/tube. The coefficient of variation was 10% within assay and 15% between assays. Further studies showed that 90–120% of added T₃ could be recovered. Usually the endogenous T₃ content of nuclear extracts was between 10 and 40 pg/0.2 ml of nuclear extract in euthyroid rats when 7 ml of the extraction buffer was used for the nuclei from 7 g of tissue, compared with less than 3.125 pg/0.2 ml in thyroidectomized rats.

Comparison of direct endogenous T₃ radioimmunoassay method with other radioimmunoassay methods after extraction. The endogenous nuclear T₃ values obtained by the radioimmunoassay after separating the endogenous T₃ and the receptor with a small column of Sephadex G-25 (Nakamura *et al.*, 1979a) were compared with those obtained by the direct radioimmunoassay method described above. As shown in Fig. 3, similar values were obtained on selected samples by both methods ($r = 0.951$ and $P < 0.005$).

Re-evaluation of nuclear T₃ receptor binding assay conditions for obtaining binding characteristics by Scatchard analysis

Thermodependent dissociation of [¹²⁵I]T₃ bound to T₃ receptor. As most or more than half of the endogenous T₃ is bound to the receptor in a nuclear extract (Surks *et al.*, 1973; Samuels *et al.*, 1974; Torresani & DeGroot, 1975), the endogenous T₃ must be dissociated from the receptor and then reach an equilibrium with added [¹²⁵I]T₃ and the receptor in the assay mixture to obtain valid binding characteristics by Scatchard analysis. To determine the extent of the dissociation of already-bound endogenous T₃ from the receptor during the assay

Fig. 3. Comparison of endogenous nuclear T₃ radioimmunoassay values obtained by the new direct (un-extracted-sample) method described in the Materials and methods section (abscissa) compared with the endogenous nuclear T₃ radioimmunoassay values obtained by the Sephadex G-25 column method (Nakamura *et al.*, 1979a) (vertical axis)

Each assay was done in duplicate. Note that there is excellent agreement between both methods, with $r = 0.951$ and $P < 0.005$ ($n = 13$). The equation for the line is: $y = 0.898x + 1.3$.

incubation, the following simulative experiment was designed.

The T₃ receptor in a nuclear extract was prelabelled with a small amount of [¹²⁵I]T₃ and the extent of dissociation of prelabelled [¹²⁵I]T₃ was measured after the subsequent addition of an excess of unlabelled T₃ (1000 pmol/tube) to avoid the rebinding. When the receptor prelabelled with [¹²⁵I]T₃ was incubated at 4°C for 20 h, only 30% of the bound [¹²⁵I]T₃ was released. However, when prewarming at 30°C was introduced at the beginning of the incubation, the release of prelabelled [¹²⁵I]T₃ increased in accordance with the duration of the prewarming. When prewarming at 30°C was 120 min, the release of bound [¹²⁵I]T₃ reached 98% (Fig. 4). This observation demonstrates that a longer duration of prewarming is more favourable for the dissociation of the endogenous T₃ from the receptor.

Effect of prewarming of binding assay mixture on nuclear T₃ receptor binding characteristics. The influence of various durations of prewarming on the binding characteristics of the nuclear T₃ receptor in the nuclear extract from a thyroidectomized rat was studied (see Fig. 5). Note that as the duration of

Fig. 4. Effects of increasing prewarming intervals on dissociation of prelabelled $[^{125}\text{I}]\text{T}_3$ from nuclear T_3 receptor (NT_3R)

A 0.1 ml portion of a nuclear extract containing 200 μg of protein from a euthyroid rat, 0.05 ml of 25 mM-dithiothreitol, and 0.05 ml of $[^{125}\text{I}]\text{T}_3$ (0.02 pmol) in BSA-TG buffer were mixed and incubated at 4°C for 6 h; this resulted in the formation of $[^{125}\text{I}]\text{T}_3$ - T_3 -receptor complex. Then 0.05 ml of BSA-TG buffer containing 1 nmol of non-radioactive T_3 was added at the beginning of the prewarming study to avoid the rebinding of $[^{125}\text{I}]\text{T}_3$ that was released from the receptor. The reaction mixtures were kept at 30°C for various time intervals as indicated on the abscissa and then transferred to 4°C. After 20 h from the beginning of the 30°C prewarming, the bound and free $[^{125}\text{I}]\text{T}_3$ were separated by the same methods as described in the T_3 receptor binding assay used for determining binding characteristics (see the Materials and methods section) and counted for radioactivity. Non-specific binding for each point was obtained by adding 1 nmol of non-radioactive T_3 before $[^{125}\text{I}]\text{T}_3$ labelling of the receptor, and the radioactivity so obtained was subtracted from the bound radioactivity of each sample. The amount of $[^{125}\text{I}]\text{T}_3$ dissociated during the prewarming and the following incubation was defined as the difference between the bound $[^{125}\text{I}]\text{T}_3$ before the prewarming and that after the incubation; values are expressed on the ordinate as a percentage of the bound $[^{125}\text{I}]\text{T}_3$ before prewarming. Each point represents a duplicate assay. As can be seen in the Figure, there was an increase in the release of $[^{125}\text{I}]\text{T}_3$ from the receptor with increasing prewarming times, reaching almost 98% at 120 min.

prewarming increased, C_{max} and K_a decreased gradually, and with 75 min prewarming those indices were significantly smaller than those without prewarming. This indicates that too long a duration of prewarming damages the receptor binding ability. However, in order to optimize the release of the endogenous T_3 from the receptor (see Fig. 4), a prewarming for at least 50 min was considered to be

Fig. 5. Effects of prewarming of binding assay mixture on binding characteristics of nuclear T_3 receptor

The binding characteristics of the nuclear receptor in the nuclear extract from a thyroidectomized rat, containing 96 μg of protein/0.1 ml were assayed as described in the Materials and methods section, except that the duration of 30°C prewarming was varied as indicated on the abscissa. The endogenous T_3 content in the extract was less than 1.6 pg/0.1 ml and was therefore omitted from the calculation of the total T_3 amounts in the assay mixtures, which varied from 6.7 to 135 pg. C_{max} and K_a were plotted on the ordinate. The results means $\pm$ s.e.m. for assays done in triplicate. It can be seen that the K_a and C_{max} steadily decreased as the time of 30°C prewarming increased, being significantly less ($P < 0.01$) than those of zero time by approx. 20% for K_a and 7% for C_{max} at 75 min prewarming. These observations indicate that the time of 30°C prewarming should not exceed 50 min.

necessary for the standard assay procedure. Under these conditions it was anticipated that 87% of the endogenous T_3 is released and C_{max} decreases by only 6% and K_a by 16%. The selection of either a longer or shorter duration of prewarming results in either too much damage to the receptor binding ability or insufficient release of the endogenous T_3 from the receptor.

Validity of correction of binding characteristics by endogenous T_3 . To demonstrate that the correction of the binding characteristics by the endogenous T_3 content in a nuclear extract gives accurate binding characteristics, a simulative experiment was conducted (see Fig. 6). A nuclear extract from a hypothyroid rat was incubated with non-radioactive T_3 to

Fig. 6. A simulative experiment to assess the validity of correcting for endogenous nuclear T_3 in nuclear extracts

A portion (0.1 ml) of a nuclear extract obtained from a thyroidectomized rat, containing 84 μg of protein and less than 1.6 pg of the endogenous nuclear T_3 was preincubated with 20 pg of non-radioactive T_3 for 6 h at 4°C to permit formation of T_3 -bound receptor. The binding characteristics were measured subsequently as described in the Materials and methods section. The Scatchard plots were calculated from the means of triplicate assays. ● shows the Scatchard plots when the amount of the preincubated added T_3 was omitted from the calculation of the total T_3 in the assay mixture, resulting in a K_a of $10.7 \times 10^9 \text{ M}^{-1}$ and a C_{max} of 46.9 pM. ○ shows the plots when the total T_3 was corrected for the amount of the added non-radioactive T_3 in the preincubation mixture, resulting in a K_a of $33.8 \times 10^9 \text{ M}^{-1}$ and a C_{max} of 52.4 pM. ▲ indicates the data obtained when the binding characteristics of the receptor in the same extract from a thyroidectomized rat were assayed without preincubation with non-radioactive T_3 , resulting in a K_a of $35.3 \times 10^9 \text{ M}^{-1}$ and a C_{max} of 53.6 pM. Note that, when the amount of known non-radioactive T_3 present in the reaction mixture was corrected (○), these results were identical with those obtained for the true binding characteristics of the nuclear T_3 receptor as determined from thyroidectomized-rat extracts which had no exogenous T_3 preincubation (▲). On the other hand, a totally different and

permit formation of T_3 -bound receptor, and the binding characteristics were then analysed by Scatchard analysis and compared with those of the receptor in the same extract measured without T_3 pretreatment. The binding rates of the receptor in the T_3 -pretreated extract was much lower than when the extract was not pretreated with T_3 , indicating that the added non-radioactive T_3 was released from the receptor during incubation and mixed with [^{125}I] T_3 . Subsequently, when the contribution of the T_3 enrichment to the extract was omitted from the calculation of the total T_3 concentration, the K_a was markedly lower and the C_{max} slightly reduced compared with the calculated values obtained when the T_3 enrichment was not ignored. When the amount of T_3 enrichment was taken into consideration, the K_a and C_{max} were very similar to the values obtained in the absence of the T_3 pretreatment.

Binding characteristics of T_3 receptor in nuclear extract from euthyroid rats. The binding characteristics of the T_3 receptor in the nuclear extracts from four euthyroid rats were measured by using the standard assay procedure described in the Materials and methods section, and the endogenous T_3 corrected results were compared with the non-corrected ones. K_a was $27.8 (\pm 2.8) \times 10^9 \text{ M}^{-1}$ (mean $\pm$ S.E.M.) and C_{max} was 271 (± 30) pg of T_3 /mg of DNA equivalent nuclear extract when corrected for the endogenous T_3 content, and they were $18.9 (\pm 1.3) \times 10^9 \text{ M}^{-1}$ and 240 (± 17) pg of T_3 /mg of DNA equivalent nuclear extract respectively when not corrected. The effect of the endogenous T_3 correction was more prominent in K_a ($47 \pm 10\%$ increase) than in C_{max} ($12 \pm 5\%$ increase).

Discussion

A new, convenient direct radioimmunoassay method has been developed to determine endogenous nuclear T_3 content which does not require an extraction or separation procedure. The sensitivity, reproducibility and recovery rate were satisfactory, and the values obtained were comparable with those obtained by another radioimmunoassay method described for determining endogenous T_3 in a nuclear extract which uses a small column of Sephadex G-25 for separation of the endogenous T_3 from the nuclear T_3 receptor as described by Nakamura *et al.* (1979a). The Sephadex G-25 method is a time-consuming technique

invalid result was obtained without correction for the known amount of non-radioactive T_3 present in the extract (e.g., the endogenous T_3 in a nuclear extract) as shown in ●, thereby producing an entirely different result from ○ and ▲.

which also restricts the number of samples that could be processed per assay run. Also, as outlined in the Results section, we observed, when using our own T_3 antibody, that the lower limit of sensitivity for the endogenous T_3 radioimmunoassay was lower in the direct direct ANS radioimmunoassay method (3.125 pg/tube) compared with the Sephadex G-25 method (25 pg/tube).

Our studies (see Fig. 6) have reconfirmed the reports of Hamada *et al.* (1979) and Nakamura *et al.* (1979a,b) that the failure to include the contribution of endogenous T_3 in the computations of binding characteristics for the hepatic nuclear T_3 receptor does result in lower values. Although it is recognized that, under experimental conditions of hypothyroidism, the contribution of the small endogenous T_3 content in the nuclear extract may not significantly influence the binding-characteristic results, a very significant contribution occurs when normal or increased thyroid-hormone function is present. Accordingly, the endogenous T_3 content of the nuclear extract should always be routinely considered in the computation of the binding characteristics from Scatchard analysis. Failure to do so under conditions of normal or increased thyroid function will result in misleadingly low computations, particularly for K_a values.

In the present study we also examined the optimal conditions required for the release of the endogenous T_3 from the receptor. We observed that a period of prewarming was necessary to improve the assay conditions. However, too long a duration of prewarming damages the receptor binding ability, as demonstrated by the steady decline in C_{max} and K_a values (see Fig. 5). In addition, prewarming improves the release of the endogenous T_3 from the receptor to facilitate the interaction of the added [125 I] T_3 and the endogenous T_3 with the receptor. In order to minimize the amount of damage to receptor binding sites while simultaneously optimizing the release of endogenous T_3 from the receptor, a prewarming period for 50 min at 30°C was selected.

In agreement with our observations, Silva *et al.* (1977) also noted that equilibrium between added [125 I] T_3 , the endogenous T_3 and the receptor in the reaction mixture was not achieved without prewarming at 30°C for 40 min. However, their recommendations for such a prewarming period were presumptive and not substantiated by information regarding the rates of dissociation or possible damage to the receptor as we have illustrated in Figs. 4 and 5. Additional studies similar to those described in Fig. 4 showed that preincubation conditions of 3 h at 20°C released only 60% of the prelabelled T_3 from the receptor (result not shown). On the basis of these observations we did not adopt such assay conditions as proposed by Torresani & DeGroot (1975) and Hamada *et al.* (1979).

The K_a value obtained in our studies for euthyroid rats was $27.8 \times 10^9 M^{-1}$, being higher than those previously reported (Thomopoulos *et al.*, 1974; Torresani & DeGroot, 1975; Hamada *et al.*, 1979). The results of Hamada *et al.* (1979), which were obtained by using an assay method most similar to ours, was $(10.3 - 14.8) \times 10^9 M^{-1}$. Regardless of the reasons for this discrepancy between K_a values, we consider that our higher K_a values could indicate that the receptor is more sensitive to T_3 concentrations than in comparison with the other assay systems employed. When the results for C_{max} are expressed as pg of T_3 /mg of DNA equivalent nuclear extract, our data were in agreement with that reported by Nakamura *et al.* (1979b) for hypothyroid rats rendered almost euthyroid after T_3 treatment. When C_{max} was expressed as pg of T_3 /mg of liver, our values of 610 ± 106 were similar to the data of Torresani & DeGroot (1975).

Fairly recently the application of Scatchard analysis to the receptor binding assay for the binding characteristics has been criticized by Seelig *et al.* (1981). These authors have proposed that weak non-specific binding sites for T_3 (other than specific nuclear T_3 receptor binding sites) could be present in a nuclear extract and that this non-specifically bound T_3 would be adsorbed on a resin because of their weak binding compared with the strong T_3 -tracer- T_3 receptor binding. Such binding sites have been termed 'inapparent non-specific binding sites'. Because of the unlimited capacity and slow backward reaction of these inapparent non-specific binding sites, it was theorized that 'free' T_3 could no longer be demonstrable after a number of hours of incubation. Studies in our laboratory (results not shown) have indicated that at least 90% of the resin-adsorbable [125 I] T_3 could be dialysed after a standard incubation of [125 I] T_3 with a nuclear extract. Hence our studies are at variance with the reports of Seelig *et al.* (1981), since we could still demonstrate a substantial amount of free T_3 available after incubation. We believe that the quasi-irreversible model proposed by those workers cannot therefore be necessarily applied to the present assay system. Consequently, although the binding characteristics obtained from the application of Scatchard analysis may be affected to some extent by the presence of the inapparent non-specific binding sites, we do not believe that this is of sufficient magnitude to invalidate the interpretation of our results. The possible influence of inapparent non-specific binding sites on the interpretation of the nuclear T_3 receptor binding characteristics obtained by Scatchard analysis is a theoretical question which will require further study.

We conclude from our studies that the measurement of the binding characteristics of the hepatic nuclear T_3 receptor can be improved by: (i)

correcting for the total T_3 content in the incubation assay mixture by adding the concentrations of endogenous nuclear T_3 determined from our new unextracted-sample radioimmunoassay method, and (ii) ensuring the validity of the Scatchard analytical results, e.g. by using the proposed preincubation warming conditions at 30°C for 50 min when performing the receptor binding assay.

We appreciate the excellent technical assistance of Mrs. Edith Gera, Mr. Paul Fung and Mrs. Marilyn Grant in the performance of this work, and Ms. D. Cartwright for expert typing assistance in the preparation of the manuscript. T. Y. was supported by a Fellowship from the Mount Sinai Hospital Department of Medicine Research Fund.

References

- Burton, K. (1967) *Methods Enzymol.* **12B**, 163–166
- Chopra, I. J., Ho, R. S. & Lam, R. (1972) *J. Lab. Clin. Med.* **80**, 729–732
- DeGroot, L. J. & Torresani, J. (1975) *Endocrinology* **96**, 357–369
- Hamada, S., Nakamura, H., Nanno, M. & Imura, H. (1979) *Biochem. J.* **182**, 371–375
- Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- Koerner, D., Surks, M. I. & Oppenheimer, J. H. (1974) *J. Clin. Endocrinol. Metab.* **38**, 706–709
- Nakamura, H., Hamada, S. & Imura, H. (1979a) *Biochem. J.* **182**, 377–382
- Nakamura, H., Hamada, S. & Imura, H. (1979b) *Biochem. J.* **184**, 143–148
- Oppenheimer, J. H., Koerner, D., Schwartz, H. L. & Surks, M. L. (1972) *J. Clin. Endocrinol. Metab.* **35**, 330–333
- Oppenheimer, J. H., Schwartz, H. L., Koerner, D. & Surks, M. I. (1974a) *J. Clin. Invest.* **53**, 768–777
- Oppenheimer, J. H., Schwartz, H. L. & Surks, M. I. (1974b) *Endocrinology* **95**, 897–903
- Samuels, H. H. & Tsai, J. S. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3488–3492
- Samuels, H. H. & Tsai, J. S. (1974) *J. Clin. Invest.* **53**, 656–659
- Samuels, H. H., Tsai, J. S., Casanova, J. & Stanley, F. (1974) *J. Clin. Invest.* **54**, 853–865
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Schmidt, G. & Thannhauser, S. J. (1945) *J. Biol. Chem.* **161**, 83–87
- Schneider, W. C. (1957) *Methods Enzymol.* **3**, 680–684
- Seelig, S., Schwartz, H. & Oppenheimer, J. H. (1981) *J. Biol. Chem.* **258**, 2154–2161
- Silva, E. S., Astier, H., Thakare, U., Schwartz, H. L. & Oppenheimer, J. H. (1977) *J. Biol. Chem.* **252**, 6799–6805
- Surks, M. I., Koerner, D., Dillmann, W. H. & Oppenheimer, J. H. (1973) *J. Biol. Chem.* **248**, 7066–7072
- Thomopoulos, P., Dastugue, B. & Defer, N. (1974) *Biochem. Biophys. Res. Commun.* **58**, 499–506
- Torresani, J. & DeGroot, L. J. (1975) *Endocrinology* **96**, 1201–1209