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HUMAN THYROXINE-BINDING GLOBULIN AND THYROXINE-BINDING PRE-ALBUMIN: DISSOCIATION RATES

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SUMMARY

1. A simple method is described for estimating the rate of thyroxine dissociation from binding sites in plasma.

2. At tracer concentrations in human plasma thyroxine dissociates at two distinct exponential rates. These are due to the two thyroxine-binding proteins present in human plasma.

3. The $t_{\frac{1}{2}}$ for thyroxine dissociation from thyroxine-binding globulin is $8{\cdot}1 \pm 0{\cdot}7$ (S.E.) min at room temperature and $38{\cdot}6 \pm 2{\cdot}1$ sec at 37° C.

4. The $t_{\frac{1}{2}}$ for thyroxine dissociation from thyroxine-binding pre-albumin is $53 \pm 5{\cdot}0$ sec at room temperature and $7{\cdot}9 \pm 0{\cdot}3$ sec at 37° C.

5. Dissociation of thyroxine from both proteins is a single exponential process, suggesting that each protein has only one type of specific binding site.

6. These results indicate that the minute pool of free thyroxine in plasma is turning over about 100 times each second, with about two thirds of this flux being due to release and binding of the hormone by pre-albumin.

INTRODUCTION

At normal concentrations almost all of the thyroxine in human plasma is bound to two specific binding proteins, viz. 60–70 % to thyroxine-binding globulin (TBG) and 30–40 % to thyroxine-binding pre-albumin (TBPA) (Brown-Grant, Brennan & Yates, 1969). A simple model has been proposed for thyroxine uptake by cells in which the hormone is released from these proteins into the free state and is then trapped by the tissues (Hillier,

1969, 1970). An integral part of this theory is a very rapid turnover of the minute pool of free thyroxine in plasma (i.e. rapid dissociation of the hormone from the TBG and TBPA binding sites). A method has already been described for measuring the total thyroxine dissociation rate in plasma (Hillier, 1970). Although it indicated rapid dissociation, the results were confined to the first few seconds of the dissociation reaction only and, as a consequence, dissociation from the two separate binding proteins could not be distinguished. In this present paper a further method is reported which confirms and considerably extends the previous work.

METHODS

Principle of the method. At tracer levels, thyroxine in human plasma is bound to specific binding sites on TBG and TBPA. Therefore, when this serum is passed through a short column of Amberlite resin (which rapidly adsorbs free thyroxine) only a small proportion of the hormone is taken up (Hillier, 1970). On the other hand, at enormously high thyroxine concentrations, these binding sites are completely saturated and the hormone is either free, or in very loose non-specific association with protein. In this state the resin column captures a very much higher proportion of the thyroxine perfused through it.

When a massive amount of stable thyroxine is added to plasma which contains thyroxine labelled with ^{125}I (radio-thyroxine) at tracer concentrations, the unoccupied binding sites are almost instantly swamped. Then, gradually, the radio-thyroxine bound to the TBG and TBPA is released into the free state. However, this released hormone is not then rebound by the TBG and TBPA sites, since these are completely saturated. It therefore remains either free or very loosely adsorbed in a non-specific fashion to any protein present. The progress of this gradual release can be followed by measuring the uptake of the radio-thyroxine by short resin columns at various times after mixing. The gradual progression from the first equilibrium state (low resin-uptake at tracer levels) to the second equilibrium state (high resin-uptake at high thyroxine concentration) is limited by the rate of thyroxine dissociation from the TBG and TBPA binding sites. If dissociation takes hours then attainment of the new equilibrium will take hours; if dissociation takes only seconds then the new equilibrium will be achieved within seconds. Measurement of the transition from one equilibrium to the other gives therefore a direct estimate of the rate of thyroxine dissociation from the various binding sites in plasma to which it is normally bound at tracer concentrations.

Procedure. It was the purpose in all experiments to mix a large amount of stable thyroxine with plasma (or with TBG and TBPA fractions of plasma) containing radioactive hormone at tracer concentrations; and then to measure the proportion of the radio-thyroxine taken up by resin columns at various times after mixing. Two procedures were adopted (Fig. 1).

1. First method. This was suitable for mixing times in excess of 30 sec and was used for studies at room temperature (20° C). Columns of resin (vol. 0·85 ml.) were perfused with 1 % human plasma in 0·1 M phosphate buffer pH 7·4 at a rate of 4 ml./min. At this rate fluid was within the column for 5 sec. Aliquots (0·1 ml.) of a solution of radio-thyroxine in 1 % plasma (or in TBG and TBPA fractions of plasma) were injected into the perfusion stream using a mixing chamber placed 2 sec before the start of the column. Injection took about 2 sec. The columns were then perfused for a further 60 sec before the resin was removed for estimation of its radioactivity.

In those experiments where the binding sites were first saturated, stable thyroxine was made up to a final concentration of 10 μg/ml. (equivalent to 1000 μg/ml. whole plasma). The concentrations needed to saturate fully the TBG and TBPA sites are 0·2 μg/ml. and 2 μg/ml. whole plasma respectively (Brown-Grant *et al.* 1969). This high concentration was therefore very much more than adequate. Mixing was done by squirting 2 ml. of the original 1% plasma (or the TBG and TBPA fractions) containing radio-thyroxine (0·05 μg/ml. whole plasma) into 20 μl. of 50% propylene glycol in water containing 20 μg of stable thyroxine (the propylene glycol helped to dissolve the hormone which is very insoluble in water). At various time intervals after this operation aliquots (0·1 ml.) of the mixture were injected into the column in the usual way. The time interval allowed for dissociation

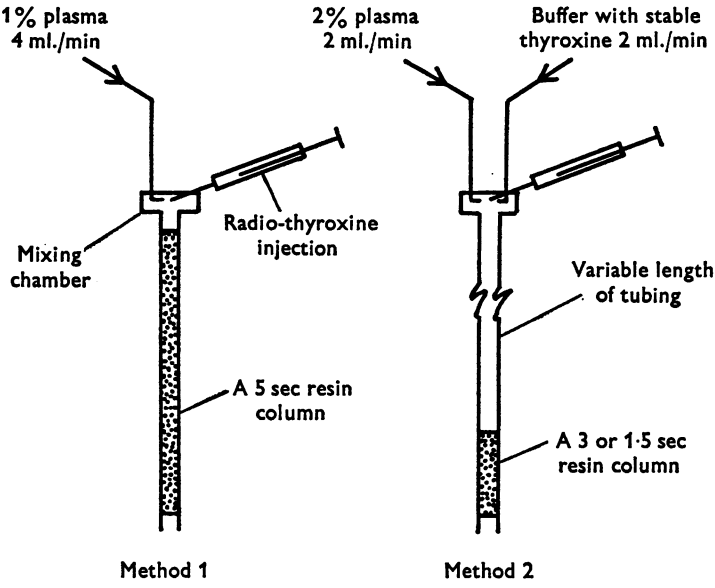

Fig. 1. The two experimental procedures employed.

was measured between the time at mixing and the time at which the radioactive solution issued from the end of the resin column (since dissociation still proceeds while the solution is in the column). The shortest practicable time interval between mixing and column exit was 30 sec.

2. Second method. This method was suitable for mixing times between about 5 and 40 sec and was used at 37° C where the dissociation rates were very fast. Stable thyroxine and human plasma were continually mixed together to give a final concentration of 1% plasma and 10 μg thyroxine/ml. (the solution of stable thyroxine contained 1% propylene glycol). This mixture was then passed through a very short resin column – a 3 sec column for the TBG experiment and a 1·5 sec column for the TBPA experiment. By putting different lengths of tubing between the mixing chamber and the column a variable delay was introduced between mixing and column exit. Aliquots (0·1 ml.) of the TBG and TBPA fractions containing tracer amounts of radio-thyroxine were injected into the mixing chamber and the columns perfused for a further 60 sec before estimation of their radioactivity. In order to determine the uptake of thyroxine at tracer levels (i.e. before mixing with stable

thyroxine) the perfusion was run initially using 2% plasma and ordinary buffer as the two solutions. In order to determine the uptake of thyroxine after equilibration at the high concentration, radioactive aliquots of the TBG and TBPA fractions which had been mixed with stable thyroxine at a concentration of 10 μg/ml. for at least an hour were injected into the mixing chamber.

Experiments with TBG and TBPA. The fractions obtained by electrophoresis (see Appendix) were diluted with 3 vol. 0·2 M phosphate buffer pH 7·4. This gave a final concentration of TBG and TBPA of about 5·5% and 4·5% respectively of the normal plasma level. These solutions were then labelled with tracer amounts of radio-thyroxine and the dissociation reactions studied in exactly the same way described previously for 1% human plasma (the columns were perfused with 1% human plasma and the final thyroxine concentration was again 10 μg/ml.).

Other materials and methods were identical to those used in a previous study (Hillier, 1970).

RESULTS

Experiments with human plasma at room temperature

Five experiments were performed at room temperature and the results of the first experiment are illustrated in Fig. 2. Stable thyroxine was added to 1% plasma containing tracer amounts of radio-thyroxine and, after various time intervals, measurements were made of the percentage of the radioactive hormone captured by a 5 sec resin column. The ordinate axis in Fig. 2 is inverted for better comparison with Fig. 3. At zero time (i.e. before mixing) the resin captured only 5·5% of the hormone, whereas about 40 min after mixing, when the second equilibrium had been reached, some 46·5% was captured. The reaction was complete at about 30 min and was extremely rapid over the first minute. In order to study the progress of the reaction more closely and, in particular, to bring out any exponential components, the results were replotted. The initial equilibrium position, the upper dashed line in Fig. 2, was designated 100% (i.e. 100% of the hormone specifically bound to TBG and TBPA) and the second equilibrium position, the lower dashed line, was designated 0% (i.e. no hormone specifically bound). A smooth line was placed through the experimental points and, using values from this line, the results were expressed on a semi-log plot (Fig. 3). By this procedure the reaction resolved into two exponential processes which almost certainly represent dissociation of the radioactive hormone from two major binding sites in human plasma.

Results collected from five experiments indicated that some 48% of the thyroxine dissociated rapidly with half-times of between 41 and 60 sec and the remaining hormone dissociated more slowly with half-times of between 5·5 and 8·0 min.

A further five experiments of this type (Method 1) were performed at 37° C but it was clear that both dissociation reactions were virtually complete at 3 min (and were therefore too fast to study accurately by this

method). These reactions were therefore exceedingly sensitive to changes in temperature and this factor certainly contributed to the variation obtained at room temperature (which fluctuated between 18 and 22° C from day to day).

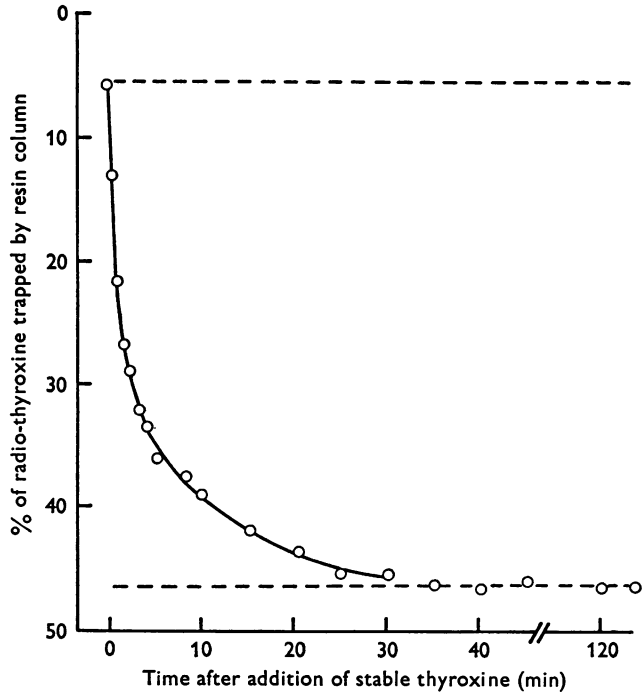

Fig. 2. Stable thyroxine was mixed with 1% human plasma containing tracer radio-thyroxine and, at various time intervals thereafter, measurements were made of the percentage of the radioactive hormone captured by a 5 sec resin column. The upper dashed line is the uptake value before addition of the stable hormone and the lower dashed line is the uptake value after equilibration at the high concentration. The ordinate axis is inverted. Each point represents a single observation and the smooth line was drawn by eye. Room temperature (19° C).

Experiments with TBG and TBPA at room temperature

The TBG and TBPA fractions were examined at room temperature using Method 1. With TBG only 4% of the thyroxine was captured by a 5 sec resin column at tracer concentrations, but at the higher concentration this value rose to about 50%. The dissociation reaction proceeded at a single exponential rate with a $t_{\frac{1}{2}}$ of 8·1 ± 0·7 (S.E.) min. In each of the nine experiments the results fitted a single exponential curve and one such result plotted on a semi-log scale is illustrated in Fig. 4.

With TBPA the equilibrium values were about 9% and 55% for a

5 sec column and again the reaction proceeded at a single exponential rate (Fig. 4) but this time with a $t_{\frac{1}{2}}$ of $53 \pm 5{\cdot}0$ sec (five experiments). The slow thyroxine dissociation rate obtained in plasma was therefore attributed to TBG and the fast rate to TBPA.

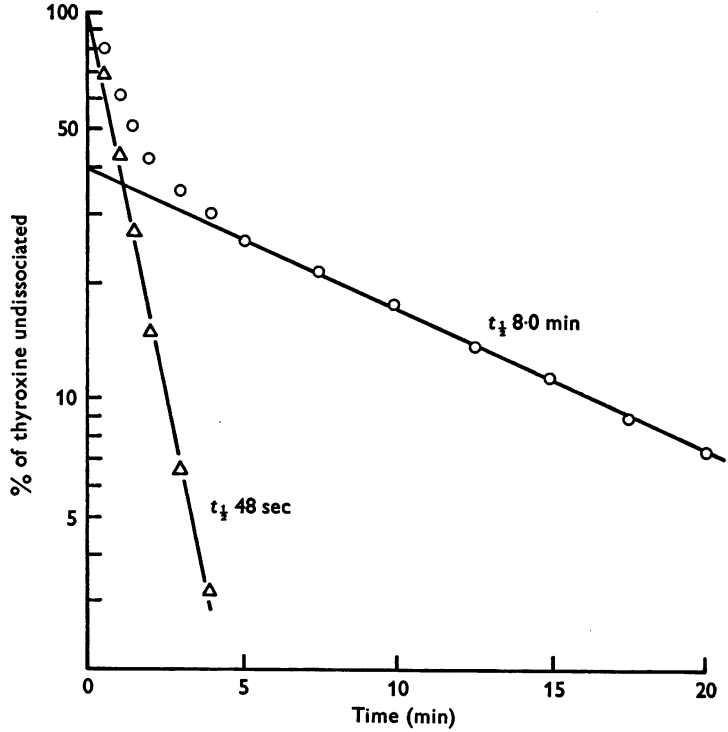

Fig. 3. The results from Fig. 2 replotted and showing the dissociation of thyroxine from two major binding sites in human plasma (for details see text). The fast exponential component (△) was obtained by subtracting the slow component (the straight line drawn through the later experimental points) from the sum of the slow and the fast components (○) during the early phase.

Experiments with TBG and TBPA at 37° C

The TBG fraction was examined by Method 2 at 37° C using a 3 sec resin column. The equilibrium values were about 8 and 60% and the reaction was again a single exponential process (Fig. 5) but this time much faster with a $t_{\frac{1}{2}}$ of $38{\cdot}6 \pm 2{\cdot}1$ sec (five experiments). For TBPA the equilibrium values with a 1·5 sec column were about 10 and 50% and the reaction again proceeded at a single exponential rate (Fig. 5) with a $t_{\frac{1}{2}}$ of only $7{\cdot}9 \pm 0{\cdot}3$ sec (five experiments). Several control experiments were performed using different dilutions of binding protein and different final concentrations of thyroxine but the dissociation rates remain unaffected.

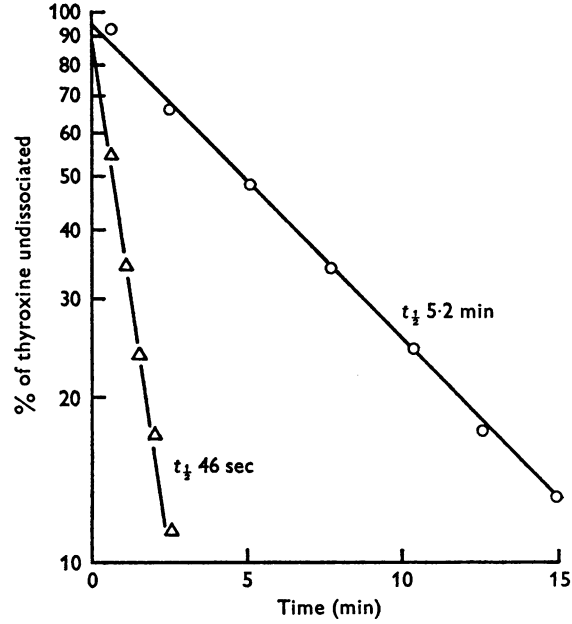

Fig. 4. Thyroxine dissociation from TBG (○) and TBPA (△) at room temperature (22° C).

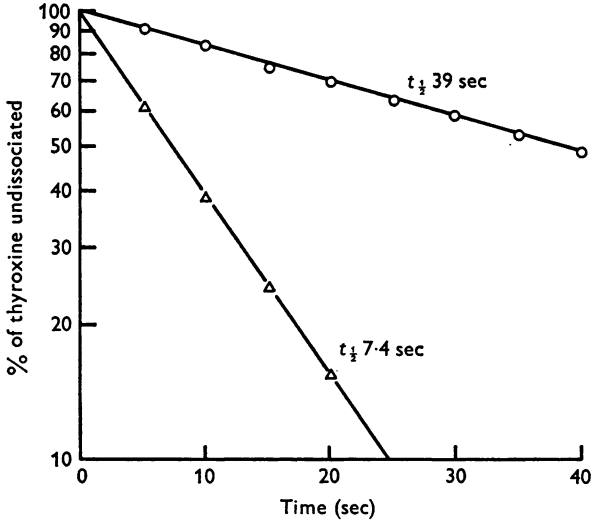

Fig. 5. Thyroxine dissociation from TBG (○) and TBPA (△) at 37° C.

DISCUSSION

These results are similar to those obtained by an earlier and quite different method (Hillier, 1970). There, the percentage of thyroxine dissociating within the first 10 sec at 37° C was estimated for bovine serum at about 22 % (human plasma was similar but was not studied in detail). In human plasma roughly 35 % of the thyroxine is bound to TBPA and 65 % to TBG and, using the half-times reported here, the corresponding dissociation for human plasma within the first 10 sec is 31 %.

With some 35 % of the hormone bound to TBPA and assuming an average value of 0·04 % for the proportion of free thyroxine (Brown-Grant *et al.* 1969) it can be calculated that, at equilibrium, the pool of free hormone is turning over about 100 times each sec. About two thirds of this flux is due to release and binding of thyroxine by TBPA.

It has often been suggested that only the free thyroxine in plasma can enter the tissues. An objection to this theory has been observation that while thyroxine uptake by tissues can be very rapid, the proportion of free thyroxine in plasma is minute (Oppenheimer, Surks & Schwartz, 1969). However, since this pool of free hormone has such a massive flux through it, rapid tissue uptake is possible provided that the tissue thyroxine-binding sites can capture the free hormone sufficiently rapidly. It is known that the liver is able to fulfil this requirement (Hillier, 1970).

APPENDIX

By W. E. Balfour

Preparation of TBG and TBPA fractions from serum

The TBG and TBPA fractions were separated from human serum by zone electrophoresis and were stored in the frozen state at −20° C until use. Details of the method have been described (Balfour & Tunnicliffe, 1960).

Serum (10 ml.) was mixed with 0·1 μg tri-iodothyronine labelled with ^{131}I. This mixture was dialysed overnight at 3° C against 2l. 0·03 M phosphate-borate buffer pH 8·4. The serum was then loaded on to a column of ethanolysed cellulose (150 × 2·5 cm) and the electrophoresis run for 48 hr at 1800 V (27 mA). Running tap water was used to cool the column during this time. After 48 hr the separated proteins were eluted from the column with the phosphate-borate buffer. The elution pattern of the protein and of the tri-iodothyronine label (indicating the position of the binding proteins) is shown in Fig. 6. Recovery of the hormone was quantitative.

Fractions 4 to 14 inclusive were combined and used as the TBPA solution and fractions 36 to 44 inclusive as the TBG solution. The concentration of

protein in the TBPA solution was 0·13 mg/ml. and in the TBG solution was 0·95 mg/ml.

It should be noted when considering the elution pattern for tri-iodothyronine that the binding of thyroxine and tri-iodothyronine in human

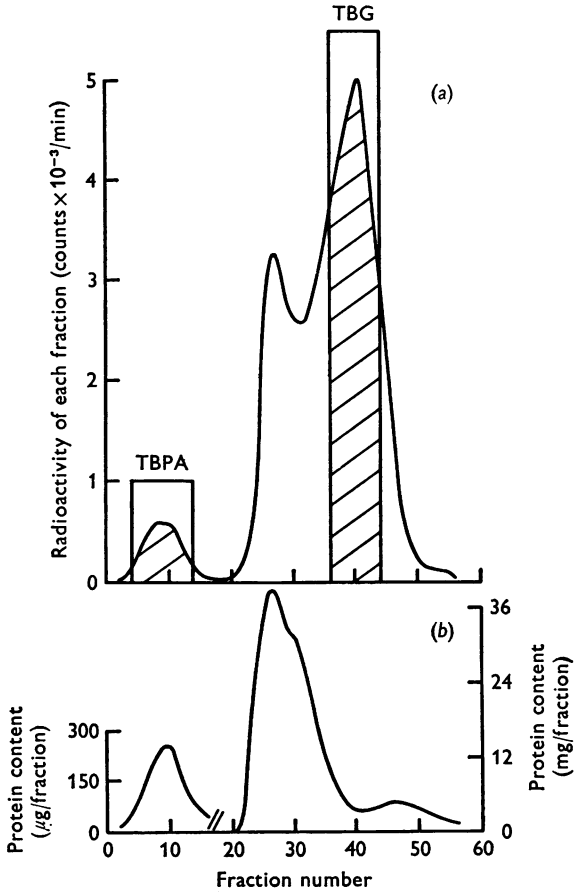

Fig. 6. The elution of (*a*) the radioactive tri-iodothyronine label and (*b*) protein from the cellulose column following electrophoresis. The protein estimations on fractions 0 to 16 were by the Folin–Ciocalteau method (left ordinate axis) and for fractions 20 to 56 by the Biuret method (right ordinate axis). Fraction volume 5 ml. The shaded areas indicate the TBPA and TBG fractions.

plasma are different. Tri-iodothyronine binds less strongly to TBG and only very weakly indeed to TBPA. Consequently a substantial proportion of this hormone (20–30 %) is found associated, probably non-specifically, with albumin (Brown-Grant *et al.* 1969).

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