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6. R. Harmel and J. Folkman, in preparation.
7. The semiautomated device for electronic readout of gelation was built for us by R. Jeffery and F. Fillippone at the C. S. Draper Laboratory of the Massachusetts Institute of Technology, Boston.

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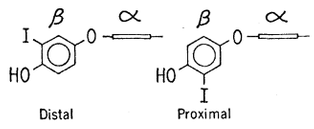
Thyroxine-Binding Globulin: Specificity for the Hormonally Active Conformation of Triiodothyronine

Abstract. *The conformational requirements for binding of triiodothyronine to thyroxine-binding globulin were investigated with triiodothyronine analogs having restricted rotation at the ether bond. Although it has been reported that the predominant conformation of triiodothyronine carries the 3′ iodine in a position proximal to the phenylalanine ring, the analog for the distal, hormonally active orientation of the 3′ iodine is more effective in displacing triiodothyronine and thyroxine from thyroxine-binding globulin. The lower binding affinity of thyroxine-binding globulin for triiodothyronine as compared to thyroxine may be explained by specificity of the binding site for the less abundant conformation of triiodothyronine.*

The single β-ring iodine of triiodothyronine (T_3) imparts a degree of asymmetry and conformational variability that is not shared by thyroxine (T_4). Both hormones must position their phenyl rings in mutually perpendicular planes to minimize steric interaction. For T_3 but not T_4, this allows two distinct conformations, one with the 3′ iodine distal to the α ring, the other with the 3′ iodine proximal to the ring. The difference between



these two conformations is of biological significance because it appears that hormonal activity is restricted to the distal orientation of the 3′ iodine (*1*). Although it seemed likely that this would prove to be the predominant conformation of T_3, x-ray crystallographic studies revealed a proximal orientation for the 3′ iodine, and this conformation was also calculated to have a lower molecular energy (*2*). Rotation at the ether bond is permitted; therefore, in free solution both conformations probably exist in an equilibrium favoring the proximal 3′ iodine orientation. However, less than 1 percent of the total T_3 in human serum is in free solution. The remainder is nonconvalently bound to thyroxine-binding globulin (TBG) and albumin.

Since the binding site on TBG shows stereochemical specificity (*3*), it was of interest to determine whether the site distinguishes the distal and proximal orientations of the 3′ iodine. Such binding specificity would strongly affect the equilibrium between the two conformations.

The conformational requirement for TBG binding was investigated with DL-3,5,-diiodo-2′,3′-dimethylthyronine as the analog for the distal 3′ iodine position and DL-3,5,-diiodo-2′5′-dimethylthyronine as the analog for the proximal 3′ iodine position. These analogs were synthesized by Jorgensen and his colleagues (*4*) to determine the relative hormonal activity of 3′ iodine in the distal and proximal positions. In these compounds rotation of the phenyl rings at the ether bond is blocked by the 2′ methyl group. Because of difficulties in synthesis, a methyl group was also introduced as the steric equivalent for the distal or proximal positions of iodine in the β ring. The distal analog is hormonally active, having 50 percent as much goiter-suppressing activity as l-T_4 and 13 percent as much calorigenic effect as l-T_3 (*5*). The proximal analog shows only 1 to 2 percent as much activity as the distal analog in these assays.

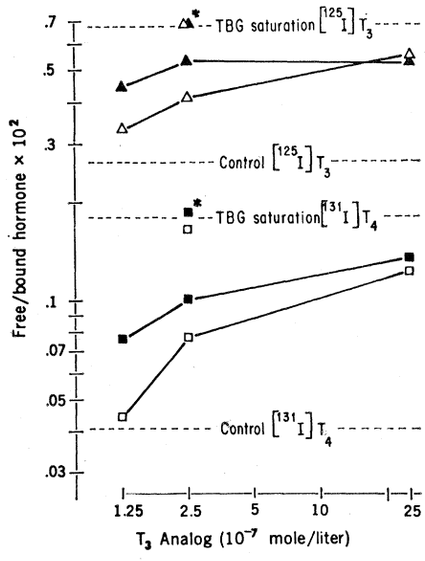


Fig. 1. Effect of analogs for the distal and proximal orientations of the 3′ iodine on ratios of free to bound T_3 and T_4 in dilute (1 : 100) human serum. For binding of [^{125}I]T_3, symbols are ▲, data for the distal analog, and △, data for the proximal analog; for binding of [^{131}I]T_4, symbols are ■, data for the distal analog, and □, data for the proximal analog. The TBG saturation lines indicate the ratios obtained when $1.28 \times 10^{-6}M$ unlabeled T_3 (enough to saturate TBG) was present in addition to the labeled hormone indicated. The pairs of data points marked by * were obtained when this concentration of unlabeled T_3 was present in addition to analog and labeled hormone. The concentrations of radioactive T_3 and T_4 were approximately $4 \times 10^{-10}M$.

The low total binding capacity of TBG (about $3 \times 10^{-7}M$ T_4 in normal human serum) made it difficult to directly measure analog bound to TBG. Therefore, the binding affinities of the oriented T_3 analogs were compared indirectly by their displacement of isotopically labeled T_3 and T_4. The T_3 analogs were dissolved in alkaline ethanol. Ratios of free hormone to bound hormone were determined by ultrafiltration of a 1:100 dilution of pooled human serum in 0.15M phosphate buffer at pH 7.4 and 37°C. This high dilution of serum increases the sensitivity of the system to competitive binding and was used to minimize the quantity of T_3 analogs required. Aliquots (25 μl) of appropriate dilutions of the stock solutions of T_3 analogs were added to 21 ml of dilute serum in phosphate buffer. An equal quantity of diluent was added to control serum. Isotopically labeled T_4 and T_3 were each separated from contaminants by a preliminary dialysis and then added to the dilute serum in a concentration no greater than 0.03 μg per 100 ml. The ultrafiltration procedure and subsequent analysis have been described (*6*). Protein electrophoresis

was carried out on paper at *p*H 8.6 in glycine acetate buffer. The distribution of $[^{131}I]T_4$ was determined by an integrating gas-flow counter and by radioautography. The paper strips were then stained to locate the protein fractions.

Electrophoretic analysis of serum containing the analogs showed that both analogs, like T_3 itself, displaced $[^{131}I]T_4$ from TBG to albumin and prealbumin. When the concentration of the distal analog was $10^{-4}M$, displacement of $[^{131}I]T_4$ was virtually complete. The proximal analog also caused marked displacement from TBG, but some TBG binding of $[^{131}I]T_4$ was retained. Electrophoresis was performed primarily to determine whether the T_3 analogs would show a binding selectivity similar to that of T_3 itself.

Quantitation of the relative effects of the distal and proximal analogs was undertaken in the ultrafiltration system. The ratio of free to bound thyroid hormones in serum was highly sensitive to the addition of oriented T_3 analogs (Fig. 1). At $1.25 \times 10^{-7}M$, the distal analog was more effective than the proximal analog in displacing both T_3 and T_4 from serum binding sites. The difference between the effects of the proximal and distal analogs decreased as saturation of the TBG binding sites was approached. Ratios of free to bound T_3 and T_4 achieved by saturation of TBG with unlabeled T_3 ($1.28 \times 10^{-6}M$) are shown (Fig. 1). As TBG becomes saturated, the ratio of free to bound hormones is increasingly determined by secondary binders, albumin for T_3 and albumin and thyroxine-binding prealbumin (TBPA) for T_4. The tendency for the analog effect on ratios of free to bound hormone to approach a limit determined by TBG saturation suggests that these secondary binders are relatively insensitive to the T_3 analogs in the range of concentrations used. This interpretation is supported by the fact that no further increase in the ratios of free to bound hormone occurred when analogs were added to serum already containing enough T_3 to saturate TBG. The albumin binding sites, which are the principal secondary acceptors for T_3, are numerous and therefore not easily saturable (*7*); but TBPA, to which T_4 was presumably displaced, is readily saturable (*8*). Therefore, the fact that the T_3 analogs had no further effect on the ratio of free to bound T_4 after TBG was saturated with T_3 indicates that the analogs, like T_3 itself, have little affinity for thyroxine-binding sites on TBPA. Reversibility of the analog effect was shown in a separate experiment; removal of the distal analog ($2.5 \times 10^{-7}M$) by overnight dialysis against 200 volumes of $0.15M$ phosphate buffer at *p*H 7.4 completely reversed the effect on ratios of free to bound iodothyronine.

Further evidence of TBG specificity for the distal 3′ iodine was obtained with the use of purified TBG (*9*), 21 μg per 100 ml of $0.15M$ phosphate buffer (*p*H 7.4), or approximately $3.3 \times 10^{-9}M$ if a molecular weight of 63,000 is assumed. This concentration was chosen to approximate the TBG concentration of the 1:100 dilution of whole serum used in the ultrafiltration experiment. Again, the distal analog ($2.5 \times 10^{-7}M$) increased the ratios of free to bound T_3 and T_4 much more than did the proximal analog at this concentration (Fig. 2). Similarly, the distal analog at $1.25 \times 10^{-7}M$ had greater effect than the proximal analog in a 1:100 dilution of bovine serum. However, in dilute rat serum neither T_3 analog was effective at this concentration.

These results indicate that the steric analogs of T_3 studied can displace T_4 and T_3 from binding sites on TBG. The effect is reversible by dialysis and requires the presence of binding sites that can be saturated by T_3. These results are explained most readily by competitive binding of the T_3 analogs to the TBG binding site for T_3 and T_4. The lack of effect of T_3 analogs in rat serum is consistent with the absence of significant T_3 binding by rat TBG (*10*). The relative effects of the two analogs on ratios of free to bound T_3 and T_4 depend on the number of iodothyronine binding sites occupied. The distal analog is more effective than the proximal analog. Thus, the orientation of the β ring preferred for binding to TBG appears to be similar to that required for hormonal activity. It was not expected that the binding preference would be absolute, because the diphenylether structure per se, independently of iodine substitution, is sufficient for weak interaction with the hormonal binding site on TBG (*6, 11*). The proportion of TBG-bound T_3 in the proximal conformation will depend on the binding preference for the distal conformation and on the ratio of proximal to distal 3′ iodine in free T_3.

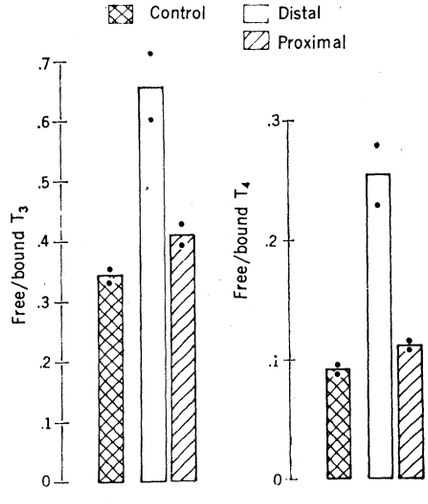


Fig. 2. Effect of oriented T_3 analogs ($2.5 \times 10^{-7}M$) on iodothyronine binding to purified human TBG (21 μg per 100 ml of buffer). The columns show average results obtained in two ultrafiltration experiments. As in whole serum, the analog for the distal 3′ iodine orientation has the greater effect on ratios of free to bound hormone.

Camerman and Camerman (*2*) pointed out that the requirement of a distal orientation of the 3′ iodine for hormonal activity could be consistent with their observation of a predominantly proximal 3′ iodine orientation if the T_3 receptor complex favors the distal orientation sufficiently (*2*). The binding of T_3 to TBG appears to be an example of such an interaction and may be a model for the interaction of the hormone with functional receptors. Furthermore, although it is usually assumed that tissue uptake occurs from free rather than bound T_3, the T_3-TBG complex may serve to carry T_3 to tissue receptor sites in the hormonally active distal conformation.

The preference of the TBG binding site for the distal 3′ iodine orientation provides an interesting if still speculative explanation for the relative binding affinities of T_3 and T_4. The former compound has an appropriate conformation for binding only when the 3′ iodine is distal, but the 3′ iodine has been reported to be predominantly proximal (*2*). On the other hand, T_4 has β ring iodines in both positions and therefore always has an appropriate conformation for binding. If the ratio of binding affinities for the two hormones depends on the relative frequency with which they present a β ring iodine in the distal position (*12*), then the relative binding affinity will reflect the molar ratio of distal to proximal 3′ iodine in T_3. This ratio is determined by the energy difference

in favor of the proximal orientation. The energy difference required to explain the observed difference in binding affinity can be calculated from the Boltzmann distribution law (*13*). At 37°C a 4 to 1 preponderance of the proximal 3′ iodine orientation, corresponding to the relative ratios of free to bound T_3 and T_4 in purified TBG solution, would be maintained by an energy difference of 854 calories. Using molecular orbital theory, Camerman and Camerman calculated a much higher energy difference (132 kcal), but they considered this likely to be an overestimation (*2*). The lower energy difference, which is consistent with the present hypothesis, would seem more likely for the coordination of the 3′ iodine with the π electron system of the phenylalanine ring, which is the probable basis for the predominance of the proximal 3′ iodine orientation (*14*). Kier and Hoyland (*15*), also using molecular orbital theory, calculated only a small energy difference that they consider to be insignificant. It is hoped that a more direct estimate of the energy difference between distal and proximal 3′ iodine orientation will be available shortly from nuclear magnetic resonance spectroscopy.

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10. Polyacrylamide gel electrophoresis of rat serum at *p*H 9.0 shows 18 percent of T_4 associated with a protein cathodal to albumin and 55 percent associated with a prealbumin, the remainder binding to albumin [P. J. Davis, S. W. Spaulding, R. I. Gregerman, *Endocrinology* **87**, 978 (1970)]. However, at *p*H 7.4 in polyacrylamide gel, T_4 is bound only to prealbumin, and T_3 to albumin. The same electrophoretic system demonstrates TBG binding of T_3 and T_4 in human serum (G. C. Schussler, unpublished observations).
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13. According to the Boltzmann distribution law the molar ratio of the two conformations of triiodothyronine will be

$$\frac{N(\mathrm{d})}{N(\mathrm{p})} = e^{-[E(\mathrm{d})-E(\mathrm{p})]/RT}$$

where d and p refer to the distal and proximal orientations of the 3′ iodine, N is the molar concentration of each such conformation, E is the total molecular energy of each conformation in calories, R is the gas constant (1.9869 cal per degree C per mole), and T is the absolute temperature.
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Wing Movements of Calling Katydids: Fiddling Finesse

Abstract. *Stridulating Uhler's katydids produce the most complex song known for insects. Series of four types of sounds are made in stereotyped sequence. Sound-synchronized high-speed photography reveals that each type of sound is produced by a distinctively different wing-movement cycle. The most complex of these cycles includes a two-step closure and a nearly silent close-open movement.*

The species-specific calling songs of male crickets and katydids (Orthoptera: Gryllidae and Tettigoniidae) have been used in studies of systematics (*1*), communication (*2*), biophysics (*3*), and neurophysiology (*4*). Such sounds are made by the scraper of one fore wing stroking the file of the other. Most species use only one type of wing-movement cycle during calling, and hence produce only a single "phonatome," that is, a single major acoustical unit corresponding to a cycle of wing movement (*5*). Their songs, then, are sequences of a single phonatome. On the other hand, some species use two or more stroking techniques during calling, and hence their songs include sequences of two or more phonatomes.

Although earlier workers have used photography to determine the relation between wing movements and the sounds produced during calling, in each case the sounds investigated suggested repetitions of a single phonatome, and a single type of wing-movement cycle was indeed found (*6, 7*). This report describes the relation between wing movements and sounds in Uhler's katydid, the only insect known to produce four phonatomes (*8*).

These katydids produce sequences of each of their four phonatomes in a stereotyped pattern lasting 8 seconds or longer. In the species studied here the calling song (Fig. 1A) begins with a sequence of 50 to 80 phonatomes of one type (hereafter designated type I). Immediately following is a sequence of 7 to 12 type II phonatomes and a sequence of 4 to 7 type III phonatomes. After a brief pause the katydid produces another sequence of 4 to 7 type III phonatomes. It may produce additional sequences of 4 to 7 type III phonatomes, but eventually it interposes one

Table 1. Comparison of four types of phonatomes produced by Uhler's katydids from Washington County, Ohio.

Phonatome type	Usual number of phonatomes in sequence	Approximate duration at 25°C (msec)	Approximate repetition rate at 25°C (sec^{-1})	Maximum sound intensity (relative)*	Amplitude of wing opening (relative)	Special features
I	50–80	70	14	0–20	50	Intensity increases during sequence; short pause during closing
II	7–12	110	9	22–26	100	Long pause during closing; nearly silent close-open
III	4–7†	45	23	28–0	100–65	Intensity decreases during sequence; wing-opening amplitude decreases during sequence
IV	3–14	20	2	18–22	20	Wing movements slight

*Based on decibel readings from a tape recorder volume-unit meter (indicates the intensity of the volume of the signal during the tape recording); agrees with subjective impression of intensity. Frequencies above 20 khz were excluded by the microphone. † More than one sequence of 4 to 7 type III phonatomes may be produced without intervening sounds.