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An Explanation for the Difference between the Responses of Mammals and Birds to Thyroxine and Tri-iodothyronine

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It is now agreed that in various biological tests in mammals, and in amphibian metamorphosis, 3:5:3′-tri-iodo-L-thyronine is about two to six times as potent as L-thyroxine (Barker, 1955). This difference in potency in mammals is maintained over a wide variety of physiological potency tests involving basal metabolic rate, body growth, serum cholesterol and antigoitrogenic action. However, Shellabarger (1955) and Newcomer (1957) reported that, in the chicken, tri-iodothyronine had the same potency as thyroxine whatever the parameter used for comparison. This is the only example of a lack of difference in the potencies of these hormones when measured by all the above tests and no explanation was produced to account for it.

In most mammals the higher potency of tri-iodothyronine is accompanied by a more rapid action, which can be attributed to a faster rate of diffusion from blood to tissues than that of thyroxine. This concept is upheld by the two to four times shorter biological half-life of tri-iodothyronine (Berson, 1956) and the comparatively feeble affinity for serum thyroxine-binding proteins (Robbins & Rall, 1957). In order to determine whether the similar responses of the chicken to the two hormones were due to similar rates of diffusion into tissues, the biological half-lives of thyroxine and tri-iodothyronine in the chicken were first determined. A study was then made of thyroxine-binding protein in chicken and duck sera, with special regard to its relative affinity for the two hormones. It was assumed that the rate of disappearance of ^{131}I from the whole body gave a satisfactory indication of the biological half-lives of the ^{131}I-labelled hormones. The results show that both thyroid hormones have similar biological half-lives in the chicken; this can be accounted for by fundamental differences between the binding of thyroxine and tri-iodothyronine by chicken- and duck-serum proteins and by mammalian-serum proteins.

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EXPERIMENTAL

Groups of five 21-day-old male chickens [Light Sussex × Rhode Island] were used for the determination of biological half-lives of L-thyroxine and 3:5:3′-tri-iodo-L-thyronine. Thyroxine (1 μg.) and tri-iodothyronine (0·82 μg.), labelled with ^{131}I with specific radioactivities of 22·1 mc and 24·3 mc/μmole respectively, were injected into each bird. The rates of disappearance of the two hormones were measured by counting the residual radioactivity in a total body ring γ-counter as described by Campbell, Cuthbertson, Matthews & McFarlane (1956), against suitable standards of each labelled compound. Measurements were made at time intervals of 5 min.–15 hr. up to 72 hr. after the intravenous injection of ^{131}I-labelled compounds. The results are expressed as the percentage of initial radioactivity found in the birds at time intervals studied.

The association of thyroid hormones with serum proteins was studied qualitatively by zone electrophoresis on paper according to methods described by Robbins & Rall (1957). Whatman no. 3 MM paper was used with 0·075M-veronal (sodium barbiturate–barbituric acid) buffer, pH 8·6 (Block, Durrum & Zweig, 1955); radioactivity was localized by an automatically recording end-window β-counter and protein fractions distributed on paper were stained with bromophenol blue. The binding of endogenously labelled thyroid hormones to serum proteins was studied in the blood of 31-day-old chickens, 24 hr. after the administration of 0·8 mc of carrier-free [^{131}I]iodide per bird. Electrophoretic analysis of such serum samples was performed only after chromatographic methods had established that 90% of circulating organic ^{131}I in butanol extracts consisted of thyroxine and tri-iodothyronine. For binding studies *in*

vitro, electrophoresis was performed on serum obtained from 21- to 31-day-old chickens and White Peking ducks (1·5 kg.). Less than 0·01 μg. of ^{131}I-labelled L-thyroxine and L-tri-iodothyronine were added to 1 ml. of serum. Human serum, under identical conditions, was used throughout this work for comparison of thyroxine-binding properties. These studies were extended to the binding of thyroid hormones to isolated serum-protein fractions. For this purpose, chicken serum was fractionated in batches of 10 ml. by a method similar to that used for human-serum proteins (Lever *et al.* 1951). Each protein fraction was dissolved in 0·15 M-NaCl at a concentration which corresponded to that in whole serum. Serum proteins were also isolated by zone electrophoresis on cellulose columns according to methods described by Flodin & Porath (1954).

Quantitative comparisons of thyroxine-binding powers of whole human and avian sera and their protein fractions were made by a method based on a recently described property of thyroxine and related iodophenols (Tata, 1959*a*). The principle of the new method involves the 'stabilization' of thyroxine and tri-iodothyronine by thyroxine-binding proteins during the ionization of their phenolic hydroxyl groups. The degree of 'stabilization' or the inhibition of the spontaneous loss of thyroxine or tri-iodothyronine is a direct function of the fraction of hormone bound and the concentration of binding proteins (Tata, 1959*b*).

The actual procedure consisted of measuring on paper chromatograms the inhibition of loss of ^{131}I-labelled thyroxine or tri-iodothyronine (as percentage of total ^{131}I) in the first 5 min. after the mixing of 0·10–0·18 μg. of the hormone, dissolved in 0·04 ml. of aqueous 20% propane-1:2-diol, with 2·5 ml. of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–maleate buffer (μ, 0·10; pH, 7·45). The inhibition of loss of radioactive material after its passage from propane-1:2-diol to the aqueous buffer was plotted against protein concentration when increasing amounts of avian or human serum or their protein fractions were added to the buffer. The protein concentrations required to produce binding or 'stabilization' of 50% of thyroxine were compared to obtain relative binding powers. At high protein concentrations there was agreement between the results obtained by this method and those obtained by the conventional electrophoretic method of displacement of bound thyroxine by saturation of the binding protein. The relative intensities with which the different thyroxine-binding components of human and avian sera bind the two hormones were measured by the electrophoretic method of displacement as described by Robbins & Rall (1957).

RESULTS

Average rates of loss of whole body radioactivity for ^{131}I-labelled L-thyroxine and 3:5:3′-tri-iodo-L-thyronine, injected into groups of five chickens, are shown in Fig. 1. The disappearance rates of the two labelled hormones were indistinguishable, a result which is different from that described for mammals. From data in Fig. 1, the biological half-life values ($T_{\frac{1}{2}}$) for thyroxine and tri-iodothyronine were calculated to be 22·5 ± 1·0 hr.

Since the difference in the relative binding affinities of the two compounds to serum proteins is reflected in the $T_{\frac{1}{2}}$ values for thyroxine and tri-iodothyronine in mammals, the next step was to compare thyroxine-binding by human serum and by chicken and duck sera. At first, qualitative experiments were performed in which the distribution of radioactivity was measured in human- and avian-serum proteins separated by paper electrophoresis. An example of such a comparison is presented in Fig. 2. A thyroxine-binding pattern, typical of that in most mammalian sera (Robbins & Rall, 1957), was observed for human serum; about 90% of added radioactive thyroxine migrated in a position intermediate between human α_1- and α_2-globulin (Fig. 2*A*). Less than 10% of ^{131}I was localized in human-serum albumin at low levels of added thyroxine. Under identical conditions, a different thyroxine-binding pattern was observed with chicken serum, whether radioactive thyroxine was added *in vitro* (Fig. 2*B*) or whether the distribution of endogenously labelled thyroid hormone was studied (Fig. 2*C*). In the chicken serum, over 80% of the total ^{131}I was localized in the albumin fraction, whereas only about 10% of the radioactivity could be detected in the α-globulin fraction. A qualitatively similar pattern was obtained with duck serum; further, chicken- and duck-serum albumins are also the principal binders of tri-iodothyronine when it is added in trace amounts to both chicken and duck sera.

Myant (1957) has shown that the thyroxine-binding proteins in rat serum cannot be distinguished from albumin under certain conditions of

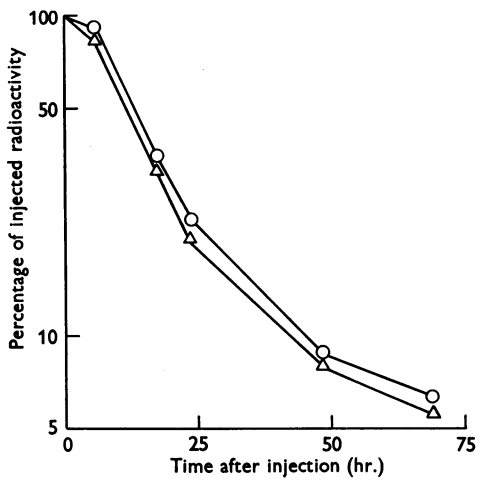

Fig. 1. Whole body disappearance rates of ^{131}I-labelled L-thyroxine and 3:5:3′-tri-iodo-L-thyronine in the chicken. Radioactive thyroxine (1 μg.) and tri-iodothyronine (0·82 μg.) were injected intravenously into groups of five birds. The residual radioactivity was counted in a total body ring γ-counter. ○, Thyroxine; △, tri-iodothyronine.

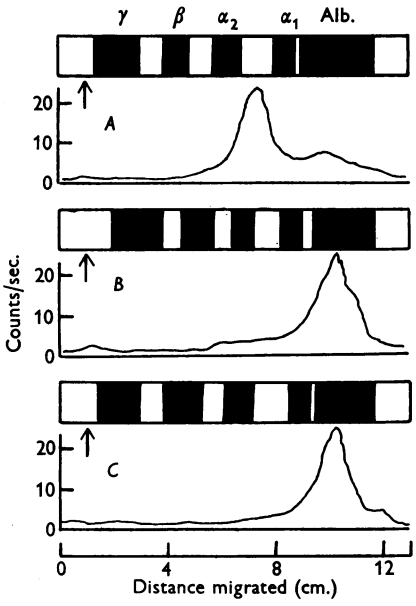

Fig. 2. Thyroxine-binding in human and chicken sera as determined by paper-electrophoretic analysis in 75 mM-veronal (sodium barbiturate–barbituric acid) buffer, pH 8·8, at 4 v/cm. for 18 hr. The bands on top show the distribution of γ-, β-, α_1- and α_2-globulin and albumin (Alb.); below them is shown the distribution of radioactivity after ^{131}I-labelled thyroxine was added to the serum or if endogenously labelled hormone was present. Vertical arrows indicate the points of application of samples. *A*, Human serum + ^{131}I-labelled L-thyroxine (0·08 μg./ml.); *B*, chicken serum + radioactive L-thyroxine (0·07 μg./ml.); *C*, chicken serum obtained from a bird 24 hr. after the injection of carrier-free [^{131}I]iodide.

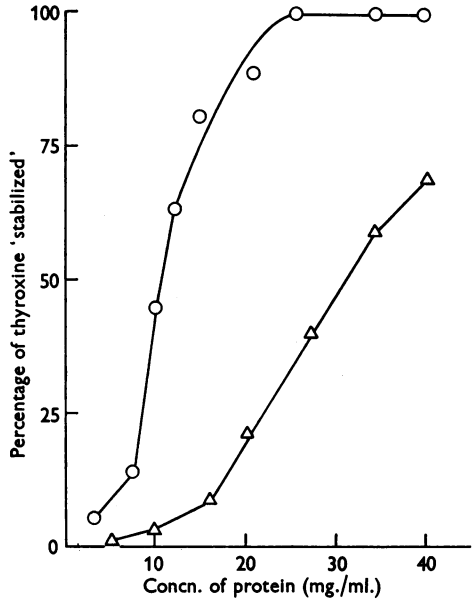

Fig. 3. Thyroxine-binding power of human and chicken sera at different dilutions. Thyroxine-binding is expressed as a percentage of thyroxine 'stabilized' at a given serum-protein concentration. 'Stabilization' was measured as follows: 0·015 μg. of ^{131}I-labelled L-thyroxine dissolved in 20% 1:2-propanediol was mixed with different amounts of human or chicken serum diluted in 0·05 M-tris–maleate buffer, pH 7·45. The percentage of inhibition (as compared with a protein-free control) of the disappearance of radioactivity in the thyroxine fraction at 5 min. after mixing in the presence of different amounts of serum was determined chromatographically. Percentage of inhibition of disappearance = percentage of thyroxine 'stabilized' or protein-bound (see Tata, 1959*b*). ○, Human serum; △, chicken serum.

Table 1. *Binding of ^{131}I-labelled L-thyroxine to protein fractions isolated from human and chicken sera*

The final concentration of added radioactive thyroxine was 63 μμM. The percentage of protein-bound ^{131}I was determined by paper electrophoresis in 0·075 M-veronal (sodium barbiturate–barbituric acid) buffer, pH 8·6.

Species	Protein fraction	Method of isolation	Concn. of protein (mg./ml.)	Percentage of ^{131}I bound to protein
Man	γ-Globulin	Ethanol fractionation	7·5	12·0–18·0*
	β-Globulin	Electrophoresis	5·0	6·0
	α_1- + α_2-Globulin	Ethanol fractionation	5·0	96·5
		Electrophoresis	5·0	100·0
	Albumin	Ethanol fractionation	15·0	42·0
		Electrophoresis	15·0	64·6
Chicken	γ-Globulin	Ethanol fractionation	7·5	8·5–15·5*
	β-Globulin	Electrophoresis	7·5	9·0
	α_1- + α_2-Globulin	Ethanol fractionation	7·5	20·8
		Electrophoresis	7·5	14·3
	Albumin	Ethanol fractionation	15·0	35·5
		Electrophoresis	15·0	52·1

* It was difficult to obtain accurate values because of the low electrophoretic mobility of γ-globulin.

Table 2. *Comparison of affinity and capacity of human and chicken sera to bind thyroxine*

Serum samples were diluted with 0·15 M-NaCl and 'stabilization' was measured in 0·05 M-tris–maleate buffer, pH 7·5, at 37° (Tata, 1959*b*).

Species	Serum dilution	Concn. of thyroxine (μg./ml.) 0·05	0·10	0·20	0·40
		Percentage of thyroxine stabilized			
Man	None	99·0	100·0	96·4	85·5
	1:2	98·0	100·0	91·2	75·0
	1:4	92·0	83·7	80·3	63·3
	1:8	57·6	47·1	28·4	23·1
	1:16	10·1	5·3	<5·0	<2·0
Chicken	None	100·0	94·5	78·2	42·2
	1:2	78·1	56·0	38·0	24·8
	1:4	28·0	22·6	10·5	8·3
	1:8	5·2	<5·0	<2·0	<2·0
	1:16	0	0	<2·0	0

Table 3. *Relative binding affinities of the major thyroxine-binding components of human, chicken and duck sera for* 3:5:3′*-tri-iodothyronine*

The values are expressed relative to the L-thyroxine-binding affinity of the particular species, taken to be 100 in each case.

Species	Sample	Concn. of protein (mg./ml.)	Relative binding affinity for tri-iodothyronine
Man	Whole serum	—	34
	Cohn fraction IV-4	7·5	30
	Prealbumin	2·0	25
	Albumin	25·0	84
Chicken	Whole serum	—	84
	Albumin	20·0	92
Duck	Whole serum	—	79
	Albumin	20·0	87

electrophoresis. In order to test further for the absence in avian serum of a thyroxine-binding protein comparable with the human protein, radioactive thyroxine was added to individual human- and chicken-serum-protein fractions after their isolation by fractionation with ethanol or by electrophoresis. As demonstrated in Table 1, chicken-serum albumin was still found to be the principal thyroxine-binding protein, whereas in human-serum proteins isolated by identical procedures the α-globulin-rich fractions were the principal thyroxine-binding fractions. Substantial binding was also exhibited by isolated human-serum albumin.

These differences in thyroxine-binding patterns were further demonstrated by the following quantitative studies: (1) a comparison of the thyroxine-binding powers of whole human and avian sera; (2) estimation of the relative affinities of the principal thyroxine-binding components of human, chicken and duck sera for thyroxine and tri-iodothyronine. Thyroxine-binding powers of whole sera were compared by measuring the fraction of thyroxine (present in a very low concentration) 'stabilized' or protein-bound in the presence of increasing dilutions of serum; this is illustrated in Fig. 3. From these results, it follows that human serum exhibits a higher binding power than chicken or duck sera. For example, if the protein concentration necessary to bind 50 % of the thyroxine is used as an arbitrary standard of comparison in data presented in Fig. 3, less human serum (10 mg. of protein/ml.) is required than chicken or duck serum (30 and 25 mg. of protein/ml. respectively) to obtain the same result. Alternatively, if the serum dilutions at which ability to bind thyroxine is lost are compared, it will be seen that for chicken and duck sera a four- to five-fold dilution was effective whereas human serum had to be diluted to one-tenth of its normal concentration. The curves in Fig. 3 cannot by themselves distinguish between 'affinity' and 'capacity' to bind thyroxine; for this purpose similar measurements were made in the presence of increasing concentrations of thyroxine (Table 2). When data from Fig. 3 and Table 2 are combined it is evident that chicken serum possesses both a lower affinity and capacity to bind thyroxine than human serum.

Comparison of the relative intensities with which human, chicken and duck sera and their constituent proteins bind thyroxine and tri-iodothyronine

revealed a further difference in thyroxine-binding in these species (Table 3). Whole human serum and its major thyroxine-binding protein components, Cohn fraction IV-4 (Freinkel, Dowling & Ingbar, 1955) and prealbumin (Schultze, Schönenberger & Schwick, 1956), all bind thyroxine about three to four times more firmly than tri-iodothyronine. In contrast, human-serum albumin, which is a secondary binding protein (i.e. the binding is evident only at thyroxine concentrations above 0·2 μM), exhibits little difference in the avidity with which it binds either hormone. Under identical conditions, whole chicken and duck sera and their serum-albumin fraction (which is their only thyroxine-binding protein component) bound thyroxine and tri-iodothyronine with nearly the same affinity. These last observations indicate the absence from avian blood of a thyroxine-binding protein capable of binding thyroxine more firmly than tri-iodothyronine.

DISCUSSION

The difference between the relative biological potencies of thyroxine and tri-iodothyronine in mammals and birds are also manifest in their disappearance rates. The identity in the biological half-lives of thyroxine and tri-iodothyronine in the chicken is the first example of its kind; in several mammalian species such as man (Sterling, Lashof & Man, 1954; Ingbar & Freinkel, 1955) and rat (Maclagan & Wilkinson, 1954; Feldman, 1957), tri-iodothyronine disappears from the blood as well as from the whole body about two to three times more rapidly than does thyroxine. The low figure of $T_{\frac{1}{2}}$ (22·5 ± 1 hr.) for the two hormones in the chicken is closer to the value for tri-iodothyronine than for thyroxine in a mammalian species of comparable body weight. The short biological half-life of thyroid hormones in the chicken may explain the low protein-bound iodine values in chicken serum reported by Mellen & Hardy (1957).

The present studies on the transport of thyroid hormones in blood show that the above-mentioned differences between mammals and birds also hold for their interaction with serum proteins. In a variety of mammals, as well as in reptiles (Robbins & Rall, 1957) and fish (Tata, unpublished work), thyroxine and tri-iodothyronine are bound in the blood to a thyroxine-binding protein fraction which is characterized by a mobility in paper electrophoresis intermediate between that of α_1- and α_2-globulin. This specific protein fraction is absent from, or barely discernible in, chicken and duck sera (Fig. 2 and Table 1). The study of thyroxine-binding by protein fractions isolated from human, chicken and duck sera by fractionation with ethanol further demonstrates this lack of thyroxine-binding protein in avian serum. Ingbar, Dowling & Freinkel (1957) showed that the human α-globulin-rich fractions, isolated by similar procedures (Cohn fractionation), constitued the most active thyroxine-binding protein fraction. Hence there exists a fundamental difference in the pattern in which thyroid hormones are distributed and bound to mammalian- and avian-serum proteins.

To give meaning to this qualitative difference in the nature of thyroxine-binding, a definition in terms of 'affinity' and 'capacity' for thyroxine-binding was necessary for a correct physiological interpretation. It is here that a useful application of a newly discovered property of thyroxine and the effect of proteins on it (Tata, 1959*a*, *b*) has been made. The data presented in Fig. 3 and Table 2 in terms of 'stabilization' of thyroxine as a measure of thyroxine-binding are interpreted to mean that the difference in binding pattern is accompanied by a lower affinity and capacity of chicken serum to interact with thyroxine than that of human serum. Further, human serum and sera of other mammalian species exhibit a markedly greater affinity for thyroxine than for tri-iodothyronine, whereas in chicken or duck serum both hormones are bound with about the same intensity. Earlier work had similarly shown the higher avidity of human serum for thyroxine (Robbins & Rall, 1955; Larson & Albright, 1955), and results presented in Table 3 further show that a selectively firmer binding of thyroxine is confined to the major thyroxine-binding protein fractions in human serum; the α-globulin-rich fraction or thyroxine-binding protein and the recently isolated prealbumin (Schultze *et al.* 1956). However, the same results also show that thyroxine-binding proteins, whether human or avian, can be divided into two classes: (1) those that bind thyroxine more firmly than tri-iodothyronine, and (2) those that bind the hormones with equal avidity. The second class consists of both human- and avian-serum albumins, indicating a common property of serum albumins irrespective of species. The principal difference between mammalian and avian sera is a lack of a specific thyroxine-binding protein in the latter.

The different metabolic and other responses exhibited by mammals and birds to thyroxine and tri-iodothyronine appear to result from the relative speeds of diffusion of these compounds from blood to tissue cells. The very similar disappearance rates of the two hormones in the chicken and the fact that they are equally strongly bound to avian-serum proteins are undoubtedly related, and the shorter biological half-life of tri-iodothyronine in mammals can similarly be accounted for by the loose nature of its binding to serum proteins compared with that of thyroxine. This supports the hypothesis that the biological potency of the

thyroid hormones in a given animal is determined by the rapidity with which they leave the blood and reach their intracellular sites of action. The study of thyroid-hormone transport in birds has therefore been valuable in revealing the physiological significance of similar data previously obtained in mammals.

SUMMARY

1. The similar potencies of L-thyroxine and 3:5:3′-tri-iodo-L-thyronine in the chicken have been further studied by comparing the overall rate of metabolism of the two thyroid hormones in both mammals and birds and by comparing binding of thyroxine by serum proteins in man, chicken and duck.

2. Thyroxine and tri-iodothyronine have indistinguishable rates of disappearance from the whole body in the chicken with half-lives of 22·5 ± 1 hr.

3. Thyroxine-binding protein as found in human serum is absent from, or present only in small amounts in, chicken and duck sera. Both endogenously labelled and exogenously labelled thyroid hormones are mostly bound to albumin in the sera of these birds.

4. Quantitative studies show that both affinity and capacity of chicken serum to bind thyroxine are lower than those found for human serum. The major thyroxine-binding protein fractions in human serum, Cohn fraction IV-4 and prealbumin, bind thyroxine about three to four times more firmly than tri-iodothyronine. Human-, chicken- and duck-serum-albumin fractions bind both hormones with almost identical intensities.

5. It is concluded that the difference in the response of birds and mammals to tri-iodothyronine, as compared with thyroxine, is due to a fundamental difference in the binding of thyroid hormones to avian-serum proteins and mammalian-serum proteins respectively.

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Inhibition of Aconitase by Glyoxylate plus Oxaloacetate

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Previous experiments showed that glyoxylate incubated with oxaloacetate in homogenates of rat liver and other animal tissue (D'Abramo, Romano & Ruffo, 1957*a*, *b*; 1958) produced a complete inhibition of citrate oxidation. The inhibition was explained by supposing that the two substances might react together to form a compound of six carbon atoms, possibly oxalomalic acid, which, even at very low concentration, could inhibit citrate oxidation by competition with aconitase or *iso*-citric dehydrogenase. Of these, aconitase was the first enzyme tested for the possibility that it could be inhibited by a condensation compound formed during the incubation of glyoxylate with oxaloacetate.

The results of these experiments show that a complete inhibition of citrate formation from *cis*-aconitate occurred in both crude and purified