Plasma Protein Binding, Half-Life, and Erythrocyte Uptake of Thyroxine and Triiodothyronine in Chickens.*† (29323)

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It has been repeatedly demonstrated in various mammalian species that triiodothyronine (T-3) exhibits 5 to 7 times the biological potency of equimolar quantities of thyroxine (T-4)(1). In the chicken on the other hand, it has been reported that T-3 is no more potent(2) or less potent(3,4) than T-4 with respect to antigoitrogenic action in thiouracil-treated chicks, and less potent in stimulating oxygen consumption of surviving cardiac muscle(5). Tata and Shellabarger (6) reported (a) the absence of a specific thyroxine-binding protein from chicken and duck sera and (b) that serum albumin fractions of chicken and duck blood bind T-4 and T-3 with almost identical intensities. They offered the suggestion that the difference in biological potency of T-4 and T-3 in mammals in contrast to chicks is due to differences in serum-protein binding ability.

Although the data of Tata and Shellabarger (6) and Shellabarger and Tata(7) apply to a comparison between mammals and birds their data do not offer a solution to the observation that T-4 exhibits greater biological potency than T-3 in chickens(3-5).

In view of the subsequent findings of Balfour and Tunnicliffe(8) of a pre-albumin component which binds T-4 in the duck and of Taurog(9) relative to the technique used by Tata and Shellabarger(6), it is desirable to obtain additional information with which to evaluate the question of relative potencies of T-4 and T-3 in chicks.

Materials and methods. Six- or 16-week old White Leghorn cockerels were fed *ad libitum* on a standard commercial chick ration and had free access to water. I^{131}-labeled T-4 and T-3 (Abbott Laboratories) with mean specific activities of 34.1 and 26.4 mc/mg respectively were used as substrates. Neither hormone was used after one week of storage at 4°C without purification by paper chromatography.

For estimation of protein-bound iodine (PBI) 6-week old cockerels were injected intravenously (i.v.) with 0.75 μg of I^{131}-labeled T-4 or T-3. At various time intervals, blood was withdrawn (never from a previous donor) and PBI estimated by the use of 1.5-2.0 cm columns (10.5 mm diameter) of a commercial ion exchange resin (Ioresin, Abbott Laboratories).

Results were expressed as

$$\frac{\text{Counts/min in effluent from column}}{\text{Counts/min in original plasma sample}} \times 100.$$

An aliquot (0.01 ml) of the effluent was subjected to paper electrophoresis in 0.075 M barbiturate buffer, pH 8.6 (180 volts, 5 milleamperes, 14 hours). Radioactive plasma protein components were located by means of an automatic recording chromatogram scanner.

For the erythrocyte uptake studies, the technique of Hamolsky *et al*(10) was used together with a slight modification of their techniques for studying the effect of various plasma dilutions on labeled T-4 and T-3 uptake.

The percent red blood cell uptake of T-3 or T-4 was calculated as follows:

$$\frac{\text{Net counts (washed RBC)} \times 100 \times 100/\text{hematocrit}}{\text{Net counts (whole blood)}}.$$

Half-life studies of T-4 and T-3 in plasma and cardiac tissue were made in 5 groups of 6-week old cockerels following i.v. injection of 1.3×10^{-3} μmoles of I^{131}-labeled T-4 or T-3. Small blood samples were withdrawn successively from the same chicks and the plasma was counted in a well-type scintillation counter. Several chicks in an additional group were sacrificed at various times after injection of labeled T-4 or T-3 (Fig. 4) and their hearts were removed; half-lives of

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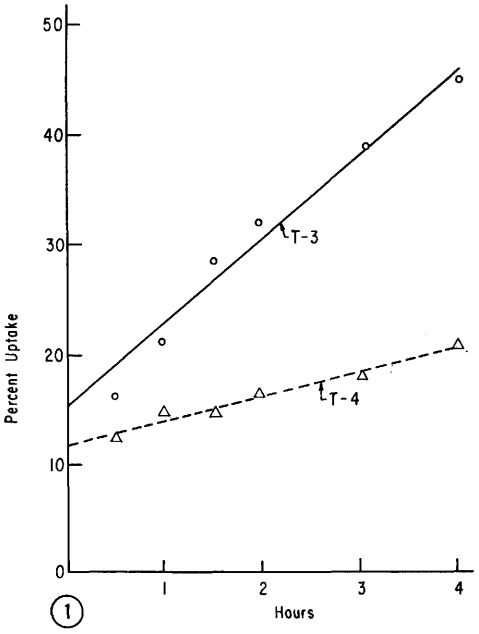

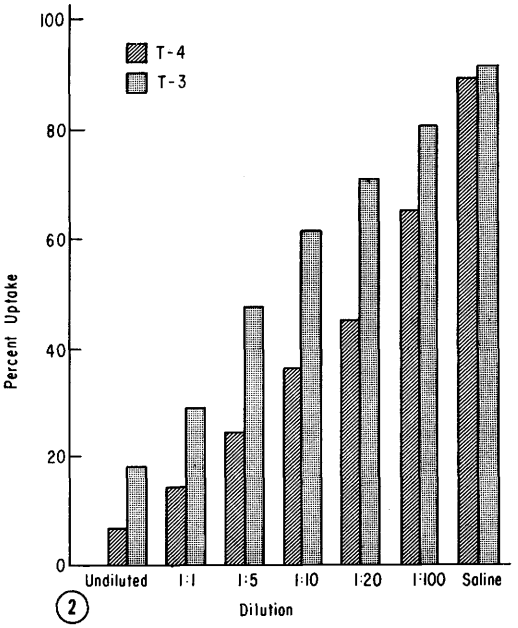

FIG. 1. Uptake of I^{131}-labeled T-4 and T-3 by avian erythrocytes.
FIG. 2. Effect of diluted plasma on avian erythrocyte uptake of I^{131}-labeled T-4 and T-3.

the 2 hormones in plasma and cardiac tissue were calculated.

Results. Binding of T-4 and T-3 to plasma proteins. Protein-bound iodine determinations were made on plasma samples at various times following i.v. injection of equimolar amounts of labeled T-4 or T-3. Approximately twice as much T-4 was bound to protein as T-3 (Table I). Since the administered dose was within the physiological range there was little chance of saturation of protein binding sites, which potentially could result in erroneous values. To demonstrate that the experimental procedure was measuring "bound" and not "free" hormone, pure, labeled T-4 or T-3 in buffer solution was added to the column; 98-99% of the material remained on the column through several washes with saline. In addition, electrophoretic analyses were made on the plasma effluent from the Ioresin columns. The results indicated that, in veronal buffer, all the radioactivity migrated with the albumin fraction, which according to various authors, is the primary site of binding of thyroid hormones to avian plasma under such circumstances (6,8).

TABLE I. Protein-Bound Iodine (% of Total I^{131}) in Plasma at Various Times After Injection of Labeled Hormone.

Time (hr)	T-4	T-3
1	54.4 ± 1.6*	30.1 ± 1.0
3	48.1 ± 1.0	36.1 ± 1.7
6	43.1 ± 3.4	17.3 ± 2.9
9	59.1 ± 1.6	26.1 ± 1.4

* Mean of 6-8 samples ± standard deviation.

Erythrocyte uptake. To substantiate the foregoing observation of differences in protein binding of thyroid hormones in chicken plasma, experiments were performed on the *in vitro* uptake of T-4 and T-3 by chicken red blood cells. It has been observed that this phenomenon reflects the affinity and/or capacity of plasma proteins for thyroid hormones; that compound having the weakest affinity (or bound less firmly) would have the greatest *in vitro* uptake by the erythrocytes(11,12,13). Results of these experiments are summarized in Fig. 1. During the 4-hour incubation period, uptake of each steadily increased. These values are considerably higher than those reported for human red cells(14). From the results of this experiment it is obvious that T-3 is taken up by the cells to a greater extent and at a faster rate than T-4.

TABLE II. Uptake of I^{131}-Labeled T-4 and T-3 (% of Total I^{131}) by Canine Erythrocytes Suspended in Avian Plasma.

Substrate	Incubation time (hr)	Mean uptake
T-4	.5	8.2
	1.0	4.7
	2.0	5.2
	3.0	5.4
	4.0	8.2
T-3	.5	5.6
	1.0	5.9
	2.0	9.4
	3.0	11.7
	4.0	15.9

If protein binding is the main factor regulating the *in vitro* uptake of the hormones by red blood cells, dilution of the plasma proteins should result in an increase in uptake. Fig. 2 shows the effects of various plasma-saline dilutions on red blood cell uptake during an incubation period of 4 hours. Quantitative values in undiluted plasma are lower than those seen in Fig. 2 due to the modified technique used in this latter study. As the plasma was progressively diluted, uptake of both hormones increased; the relative percent increase was greater for T-4 than T-3. This latter finding would be expected if more T-4 than T-3 was bound to the proteins. When only saline was used as the red cell suspending medium, uptake by the cells was identical for both hormones. These findings agree very well with those reported by Friis (13) and Crispell *et al*(11) in mammals.

Since the possibility arose that chicken red cell uptake may be affected by the presence of the nucleus, non-nucleated cells (dog) were incubated with chicken plasma to see if any qualitative difference in response would occur (Table II). As one would expect, extensive hemolysis occurred which yielded lower uptake values than normal, nevertheless, in the one experiment that was conducted, the canine red blood cells took up the T-3 to a greater extent and at a faster rate than they did the T-4.

Half-life in plasma and cardiac tissue. Table III summarizes half-life determinations of T-4 and T-3 in the plasma of 16-week old cockerels during various time intervals following intravenous injection of each hormone. Time interval A is characterized by a half-life of approximately 3.0 minutes for both hormones; time intervals B and C exhibit decay rates characterized by a half-life of approximately 29.0 minutes for both hormones; time interval D has a characteristic half-life of 7.2 hours for T-3 and 8.3 hours for T-4 ($p<0.6$); time interval E is characterized by a half-life of approximately 16.4 hours for both hormones.

In cardiac tissue, T-3 had a mean half-life of 3.9 ($\pm$ S.E. of 0.7) hours; T-4, 4.9 ($\pm$ S.E. of 0.7) hours (based on 5 determinations for each). This difference was statistically significant at the 94% level.

Discussion. The study of the protein-binding of thyroid hormones to chicken plasma proteins by the methods employed here reveals the same relationship as the protein binding of thyroid hormones in mammalian serum *i.e.* T-4 is bound more extensively and firmly than T-3. The criterion of binding affinity used by Tata(15) relates to the transient instability of I^{131}-labeled T-4 and other iodophenols being inhibited by the presence of human serum in aqueous medium; the amount of T-4 "stabilized" was proportional to the T-4 binding properties. By this method Tata found T-3 to be bound less firmly than T-4 in mammals, but both hormones were bound with equal intensities in chickens and ducks.

The procedure used by Tata and Shellabarger(6) should be reevaluated in the light

TABLE III. Biological Half-Life of I^{131}-Labeled T-4 and T-3 in Plasma of Cockerels During Various Time Intervals.

	Time interval From	To	T-3	T-4
A	1.0	4.0 min	3.4 min*(2) $\pm$2.8	3.0 min (4) $\pm$ .6
B	5.0	20.0 "	29.3 " (3) $\pm$6.6	28.9 " (5) $\pm$7.5
C	20.0	50.0 "	27.3 " (5) $\pm$2.4	31.1 " (5) $\pm$4.0
D	1.0	24.0 hr	7.2 hr (8) $\pm$1.9	8.3 hr (9) $\pm$1.6
E	1.0	4.0 days	16.3 " (5) $\pm$6.2	16.5 " (8) $\pm$2.0

* Mean $\pm$ standard deviation. ( ) No. of determinations.

of the failure of Taurog(9) to confirm Tata's (15) results when drying time prior to chromatography was controlled.

Although the protein-bound iodine determinations reported herein do not *per se* unquestionably prove a greater affinity of T-4 for the plasma binding sites than T-3, the higher values obtained do infer a greater affinity for the sites that are available.

More convincing are the red cell uptake data. Experiments with this technique previously have been conducted almost exclusively on red blood cells from human beings (14,16,17). Crispell *et al*(11,12,18) compared the *in vitro* uptake of I^{131}-labeled T-4 and T-3 by human erythrocytes by taking a volume of washed cells and incubating them with plasma containing the labeled hormones. In all instances a greater percentage of the T-3 was taken up (or bound) by the red cells than occurred with T-4.

The explanation was proposed that the difference in uptake of the two hormones by the red cells reflected relative binding abilities of the plasma proteins. To substantiate this hypothesis the washed red cells were incubated with various concentrations of plasma; as the plasma was diluted with saline the uptake of T-4 by the cells increased in proportion to the amount of saline added(11). In all cases the increase in uptake was greater for T-3 than T-4. Friis(13) added support to the concept of protein binding as the main mechanism responsible for the red cell uptake of T-3 by showing that the amount of T-3 taken up was increased as the serum was diluted. Addition of hormone in amounts up to 0.2 μg had no effect on red cell uptake. It was concluded that the difference in binding must be due to plasma proteins. The injection of trypan blue(18) appeared to compete with T-4 for the binding sites on TBP since this compound caused an appreciable rise in red cell uptake. The nature of the red cell uptake was thought to be a physiocochemical phenomenon not directly dependent on enzymatic processes of red cell respiration.

Interpretation of the protein-bound iodine determinations and red cell uptake values of the present experiment in terms of affinities of T-4 and T-3 for chicken plasma proteins suggests that T-3 has a markedly lower affinity for the plasma proteins than does T-4. In support of this conclusion is the work of Balfour and Tunnicliffe(8) who found that electrophoresis of duck serum on cellulose columns with borate-phosphate buffer, revealed the presence of a pre-albumin protein fraction which binds T-4 to a considerable extent. Moreover, Ingbar(19) has found that pre-albumin possesses essentially no binding power for T-3 in mammals.

The half-lives of T-4 and T-3 in plasma were found to be almost identical regardless of the time intervals used to calculate the values. Clearly, at least 4 rate constants are involved in the disappearance of T-4 and T-3 from chicken plasma up to 4 days post-injection probably representing distribution into various body iodine pools(20).

Previously, evidence was presented to indicate that T-3 has a lower affinity for chicken plasma proteins than does T-4. Accordingly, T-3 should have a shorter half-life in plasma if, indeed, protein binding is the main factor regulating the disappearance of the hormones from plasma. Roche *et al*(12) have postulated that the thyroxine-binding protein crosses directly from the capillary wall to the interstitial fluid and thus disappearance of the hormones from the blood may be independent of protein binding. Engbring and associates(22) have stated that under various circumstances, prolonged half-life of T-4 (as in myxedema) may be independent of protein-bound iodine values.

Since there appears to be no difference in the half-lives of the 2 hormones in chicken plasma (a fact also reported by Tata and Shellabarger, 6), factors other than, or in addition to, protein binding must account for the disappearance rates from the circulation. This finding has an important functional interpretation. The fact that T-3 was observed to have shorter half-life in cardiac tissue than T-4 may be one factor which could explain why T-4 is more potent in stimulating oxygen consumption in this organ(5). If T-4 remains in the tissue for a longer period than T-3, presumably it could potentially stimulate metabolic processes for a longer period

with the net result of a more potent action.

Summary. The capacity and affinity of avian plasma proteins for triiodothyronine are less than those for thyroxine. The half-lives of thyroxine and of triiodothyronine in avian blood plasma are approximately equal; the half-life of triiodothyronine in cardiac tissue is consistently less than that of thyroxine.

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Bacteria Induced Morphologic Changes.* (29324)

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The occurrence and characteristics of enlarged ceca in germfree mammals has been reviewed by Luckey(1). Enlargement of the cecum is not a constant phenomenon in all germfree animals: some germfree rats have no enlargement of the cecum and it does not occur in germfree chicks. Reduction in the size of the enlarged cecum when germfree mammals were contaminated or inoculated with normal intestinal flora has been reported repeatedly. However all bacteria do not contribute this factor since enlarged ceca were regularly seen in germfree rats inoculated with *Lactobacillus acidophilus* and their gntophoric offspring. Gordon(2) reported that the rate of weight reduction of the cecal wall was considerably slower than that of the cecal contents. This led to the assumption that the increased cecum size of germfree mammals was due to reduced muscle tone.

The poor development of the lymphatic system of germfree mammals, first noted by Glimsted(3), was also reviewed by Luckey (1). Since the lymphatic system is intimately involved in the cellular and humoral responses to infection, it is assumed that the absence of demonstrable flora is a prime factor in this underdevelopment. The classic laboratory rat has a mass of lymphatic tissue at the apex of the cecum about one centimeter in diameter. That of the classic mouse is about 2 mm in diameter. Grossly and microscopically this looks like a large Peyer's patch and was noted by Farris and Griffith(4). The apex is the only place in the cecum where a well defined patch of lymphatic tissue is seen in classic rats, mice, hamsters and guinea pigs. The appendix of man, which appears at the apex of the "residual" cecum, is a lymphatic organ. Rabbits have both structures: the distal one-

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