BINDING OF THYROID HORMONE BY ERYTHROCYTE CYTOPLASMIC PROTEINS

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SUMMARY: Characteristics of iodothyronine-binding to dog erythrocyte cytosol proteins are described. Half-time of association of both thyroxine (T_4) and triiodothyronine (T_3) is 60 min and equilibrium is achieved at 120 min (20°C). Binding is enhanced at 37°C compared to 20°C. T_4 and T_3 binding capacities of the cytosol are 10 and 5 picomoles/mg cytosol protein, respectively. Gel filtration (G-100) reveals 3 protein fractions that dissociably bind both T_4 and T_3. Quantitative and qualitative differences distinguish the erythrocyte cytosol "receptor" proteins from those previously described in other dog tissues. The erythrocyte is a model for studying functions of cytosol "receptors" for iodothyronines.

Nuclear receptor sites and actions of thyroid hormone are well-documented (1-3) and extranuclear actions have also been described. The latter may include cell glucose uptake (4), amino acid uptake (5), control of the number of cell surface adrenergic receptor sites (6, 7), synthesis of erythrocyte 2, 3-diphosphoglyceric acid (8) and putative mitochondrial actions (9). Ideally, studies of extranuclear actions of iodothyronines should be conducted in cell model systems in which nuclear binding is negligible. Such a model system would also be advantageous for studies of possible functions of receptor sites for thyroid hormone on soluble proteins (10-12). In this report we describe cytoplasmic receptor proteins for

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thyroid hormone in the mammalian erythrocyte, a nonnucleated cell upon which thyroid hormone is known to have effects (8, 13).

METHODS

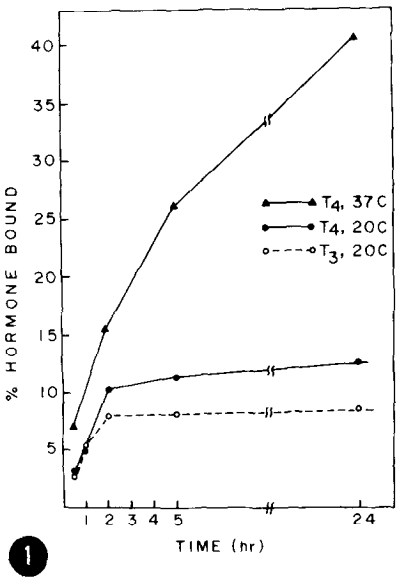
Dog blood. Awake adult mongrel dogs were bled 100 ml from foreleg veins into heparinized syringes. Blood was centrifuged X 3,000 rpm X 10 min and a 38 ml pellet was obtained.

Hemolysate and cytosol preparation. Erythrocytes were washed 3 times with 0.9% NaCl. Cells were lysed with the addition of 10 vols ice cold 0.005M Tris, 0.0004M EDTA, pH 7.6. Following rapid stirring X 20 min, the resultant mixture was spun at 20,000 X g X 20 min. The supernatant was then centrifuged at 100,000 X g X 60 min to yield "erythrocyte cytosol." Protein concentration of the supernatants was about 50 mg/ml. Cytosol was either used immediately or stored at -20°C.

Erythrocyte cytosol contained no serum proteins when reacted against rabbit anti-dog serum serum (Pentex) by immunodiffusion (2-dimensional double diffusion in buffered 1% agarose at 20°C); immunoprecipitates occurred with myocardial cytosol control and were absent from kidney cytosol control. The possibility of contamination of cytosol with serum thyroid hormone-binding proteins we have previously described (14) was also excluded by polyacrylamide gel electrophoretic studies which compared hormone binding protein mobilities and by competitive displacement analysis.[1]

Hormones. [^{125}I] Thyroxine (T_4)(48 Ci/mmol) and [^{125}I] triiodothyronine (T_3)(39 Ci/mmol) were obtained from Abbott Laboratories and their purity verified chromatographically (15). Unlabeled T_4 and T_3 were obtained from Sigma.

Iodothyronine-binding studies. Cytosol was incubated with labeled T_4 or T_3 for 5 hr at room temperature (20°C) to insure equilibration of iodothyronines with receptor sites (see below). The resultant mixture was subjected to chromatography on Sephadex G-100 as previously described (10). Dimensions of the columns were 0.9 cm X 54 cm. The



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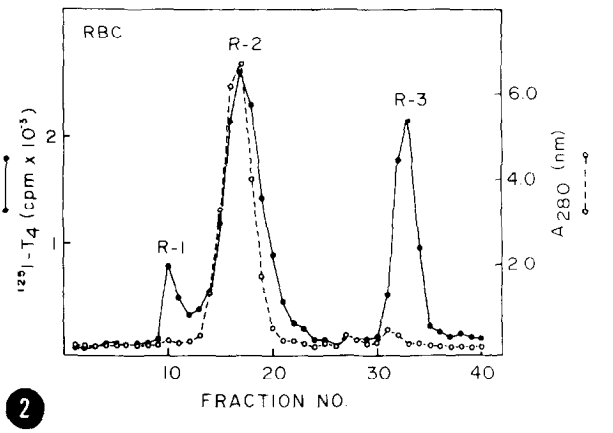

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Fig. 1: Time course of association of $[^{125}I]T_4$ and $[^{125}I]T_3$ with dog erythrocyte cytosol proteins. Bound hormone was determined by gel filtration. Concentration of iodothyronines was 1 X 10^{-9}M. Studied at 37 C, T_4-binding did not achieve equilibrium by 24 hr.

Fig. 2: Gel filtration (G-100) of erythrocyte cytosol equilibrated with $[^{125}I]T_4$ (1 X 10^{-9}M). Thyroxine emerges in three fractions (R-1, R-2, R-3). Unliganded T_4 is eluted in fractions 43-50, not shown in the figure. Fraction volume was 1.5 ml, void volume of column was 15 ml. RBC, red blood cell cytosol

by equilibrium dialysis (17), carried out at 20°C X 20 hr, or by gel filtration. Protein concentration was measured by the Lowry method (18), using bovine serum albumin as a standard.

RESULTS

Time course of association of iodothyronines with erythrocyte cytosol. Binding of T_4 and T_3 by cytosol proteins achieved equilibrium at 20C at 2 hr (Fig. 1), with a half-time of association of 60 min. Preliminary studies of binding carried out at 37°C indicate that the binding of T_4 is markedly enhanced at physiologic temperature and has not reached equilibrium by 24 hr.

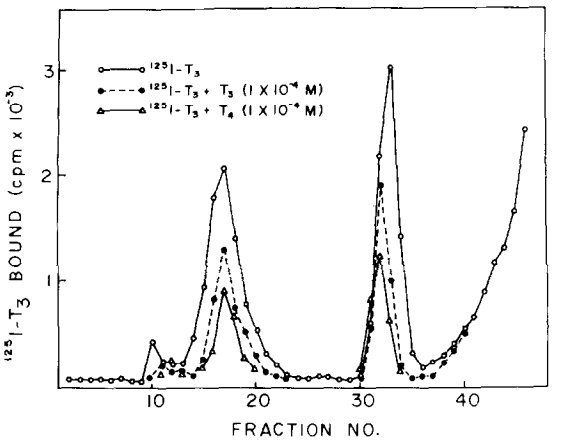


Fig. 3: Gel filtration (G-100) of erythrocyte cytosol equilibrated with [^{125}I]T_3 (1 X 10^{-9}M). Triiodothyronine emerges in same three fractions in which T_4 is eluted (see Fig. 2). Unliganded hormone is eluted in fractions 37-45. Labeled T_3 is readily displaced from peaks R-2 (fraction 18) and R-3 (fraction 33) by excess unlabeled T_4 or T_3. Some displacement also occurs from peak R-1 (fraction 10).

Gel filtration of cytosol containing labeled iodothyronines. Labeled T_4 is eluted on Sephadex G-100 in three fractions (Fig. 2). A similar elution profile was found for [^{125}I] T_3 (Fig. 3). Binding in fractions R-2 and R-3 is trypsin-digestible (1 mg trypsin/ml cytosol, incubated at 37°C X 60 min). Digestion resulted in decreases in binding of tracer by fractions R-2 and R-3 of 42% and 62%, respectively. In contrast, trypsin digestion enhanced iodothyronine-binding by R-1, a fraction which elutes with the void volume. Pronase-digestion (1 mg pronase/ml cytosol, 37°C X 60 min) had similar effects on R-1, R-2 and R-3. These findings may reflect differences in the arginine-lysine content of fractions R-1 and R-2/R-3.

Displacement of iodothyronines from cytosol binding sites. Progressive additions of unlabeled T_4 to unfractionated cytosol containing 1 X 10^{-9} M [^{125}I]T_4 resulted in displacement of the labeled hormone (Fig. 4). Displacement studies carried out on cytosol fractions R-1, R-2

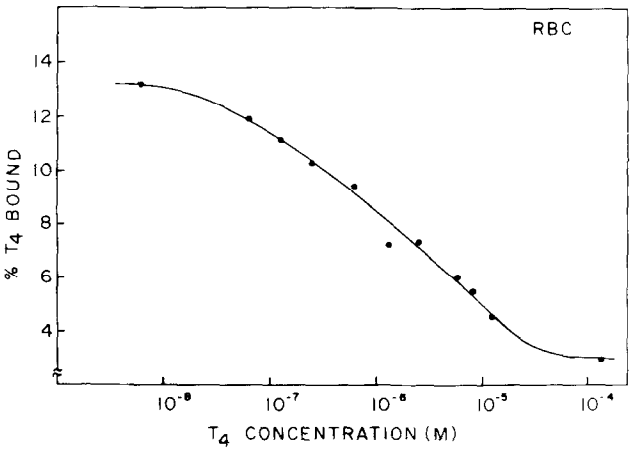


Fig. 4: Thyroxine displacement curve carried out on unfractionated erythrocyte cytosol. Bound T_4 was determined by gel filtration. Cytosol contained [^{125}I]T_4 (1 X 10^{-9}M) and various concentrations of unlabeled T_4. RBC, red blood cell cytosol

and R-3, utilizing [^{125}I]T_3 (Fig. 3) and [^{125}I]T_4 (Table 1), indicate that dissociable binding occurs in all three moieties. Unlabeled T_4 is at least as effective as unlabeled T_3 in displacing [^{125}I]T_3 from binding sites in fractions R-2 and R-3 (Fig. 3)

Applying saturation methodology to unfractionated cytosol it was possible to show a plateau in the binding of T_4 at a T_4 concentration in cytosol of 1 X 10^{-5} M (Fig. 5). Determined by this method, the binding capacity of cytosol protein for T_4 is 0.5 nmoles T_4/ml cytosol (10 picomoles/mg cytosol protein). T_3 binding capacity is 5 picomoles/mg protein.

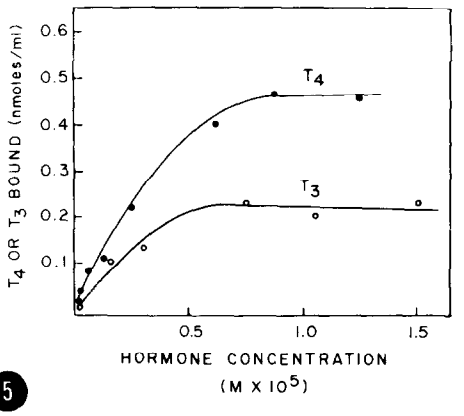
Relationship of cytosol concentration to hormone-binding. Per cent dialyzable [^{125}I]T_4 and [^{125}I]T_3 increased linearly with cytosol dilution (Fig. 6) 1:1, 1:2, 1:4). Dilutions beyond 1:8 resulted in >50% dializable T_4 and T_3 (results not shown).

Table 1

Dissociability of [^{125}I]T_4-binding by erythrocyte cytosol protein fractions

Eluted fraction	T_4 concentration	[^{125}I]T_4 eluted in fraction (cpm/fraction)	Percent reduction in binding of [^{125}I]T_4 by protein fraction
R-1	1×10^{-9} M	1266	
	1×10^{-4} M	1021	19
R-2	1×10^{-9} M	11551	
	1×10^{-4} M	2264	80
R-3	1×10^{-9} M	5395	
	1×10^{-4} M	3396	37

Fraction R-1, tubes 10-12; fraction R-2, tubes 16-19; fraction R-3, tubes 32-35. T_4 concentration of 1×10^{-9} M represents samples containing tracer, alone; concentration of 1×10^{-4} M reflects 1×10^{-9} M [^{125}I]T_4 + unlabeled T_4 to achieve the cited concentration.



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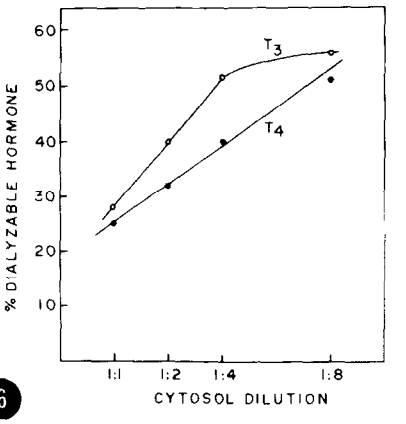

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Fig. 5: Determination by saturation methodology of binding capacities of erythrocyte cytosol for T_4 and T_3. Cytosol contained labeled T_4 or T_3 (1 X 10^{-9}M) and varying concentrations of the appropriate unlabeled iodothyronine. Cytosol protein concentration (Lowry) was 49 mg/ml.

Fig. 6: Effect of dilution of erythrocyte cytosol on dialyzable T_4 and T_3. Concentrations of T_4, as [^{125}I]T_4, and T_3, as [^{125}I]T_3, prior to dialysis were 1 X 10^{-9}M.

DISCUSSION

Several thyroid hormone effects on the mammalian erythrocyte are known (8, 13), but the sequence of cellular events leading to the expression of these actions is not clear. In nucleated cells, however, thyroid hormone has been postulated to express its effects via nucleus-directed protein synthesis (1,2). Nuclear effects of thyroid hormone are thought not to require cytoplasmic binding of hormone (19). Actions which thyroid hormone has on erythrocytes, however, must be expressed in the absence of nuclei and mitochondria.

It is well-known that iodothyronines bind to erythrocyte membranes (20,21), but earlier studies ofthe binding of T_4 and T_3 by erythrocytes have not defined soluble receptor sites. We have previously suggested in studies of liver and kidney cytosol (10,22,23) that the affinity of soluble receptor sites for T_4 is greater than that for T_3. The current studies of

erythrocyte cytosol are consistent with these previous observations in other tissues.

The erythrocyte appears to be a suitable model in which to study the relationship of soluble intracellular receptor sites for iodothyronines to actions of thyroid hormone specific to this cell. It is important to note that differences exist between dog erythrocyte cytosol iodothyronine-binding proteins and cytosol binding proteins described in liver and kidney (10, 22, 23). These differences are both qualitative and quantitative. Although liver or kidney cytosols and erythrocyte cytosol contain 3 binding protein fractions, nondissociable T_4-binding characterizes 2 of the 3 kidney and liver fractions. In addition, the capacity of erythrocyte cytosol to bind T_4, expressed per mg of cytosol protein, is 1/20 that of kidney cytoplasm (10, 23). Finally, the enhancement of T_4-binding to erythrocyte cytosol protein(s) at 37°C (Fig. 1) is absent from kidney cytosol (K. Yoshida and P. J. Davis: unpublished observations). Unique aspects of the erythrocyte "receptors" for thyroid hormone in cytoplasm, then, are consistent with a role for such receptors in actions of the hormone which are unique to the erythrocyte (8) or which can be expressed in the nonnucleated erythrocyte (13, 24) as well as in nucleated cells. The erythrocyte is also proposed as a possible model in which to study the effects of alterations in extracellular free hormone on intracellular free T_4 and T_3 concentrations.

ACKNOWLEDGMENT

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