

# Thyroid Hormone Binding by Human Serum Prealbumin (TBPA)

## ELECTROPHORETIC STUDIES OF TRIIODOTHYRONINE-TBPA INTERACTION

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**ABSTRACT** Thyroxine-binding prealbumin (TBPA) in normal human serum has been shown in a polyacrylamide gel electrophoresis system to bind 7–9% of tracer level purified [ $^{125}$ I]triiodothyronine (T3), and more than 30% of T3 in serum deficient in thyroxine-binding globulin (TBG). The T3-TBPA interaction has been confirmed at pH 9.0 and pH 7.4 in this electrophoretic demonstration of TBPA binding of T3 in serum. Purified human TBPA has also been shown to bind T3. Progressive additions of unlabeled thyroxine (T4) to serum containing tracer [ $^{125}$ I]T3 displace T3 from TBG, its principal carrier, to TBPA and albumin; however, T4 loading does not lead to significant T3 displacement from TBPA even at T4 levels known to saturate TBPA. Loading of serum with unlabeled T3 results in displacement of more than 50% of [ $^{125}$ I]T3 from TBPA, as well as from TBG, to albumin. Studies carried out with serum containing diphenylhydantoin (DPH) or MK-185, known inhibitors of T4 binding by TBG, also showed T3 displacement from TBG to TBPA and albumin. Although salicylate and tetraiodothyroacetic acid (TETRAC) displace T4 from sites on TBPA, they have only minimal effects on T3-TBPA interaction.

### INTRODUCTION

Thyroxine-binding prealbumin (TBPA)<sup>1</sup> in human serum was first described in electrophoretic studies by

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<sup>1</sup> *Abbreviations used in this paper:* DPH, diphenylhydantoin; MBC, maximum binding capacity; MK-185, 2-acetoamidoethyl(4-chlorophenyl)(3-trifluoromethyl phenoxy)ace-

Ingbar (1). Prealbumins which bind thyroxine (T4) are also present in certain animal sera (2–4). It has been generally acknowledged that human TBPA does not bind triiodothyronine (T3) (5–9). Recent emphasis on the metabolic importance of serum T3 (10–12), coupled with our own observations that animal serum prealbumin may bind T3 (13), caused us to investigate T3 binding in human serum using previously described polyacrylamide gel electrophoresis methods (4, 14). We find that TBPA in normal human serum can indeed be shown electrophoretically to bind T3.

### METHODS

**Sera.** Sera were obtained from three healthy euthyroid adult male subjects (N. B., R. S., P. D.) and from male patients with no immunologically demonstrable thyroxine-binding globulin (TBG) (J. W.) or with low TBG levels (G. G.).<sup>2</sup> Sera were also obtained from four additional normal adults and pooled for certain studies at pH 7.4. Hypothyroxinemic serum (0.1  $\mu$ g thyroxine (T4) per 100 ml, by column chromatography) was available from a patient with untreated primary hypothyroidism (D. S.). All sera were stored at  $-17^{\circ}\text{C}$  until used.

**Radioactive hormones, chemicals, and biological materials.** [ $^{125}$ I]T4 was obtained from Abbott Laboratories (North Chicago, Ill.). It contained 91% T4 in a silica gel thin-layer chromatography (TLC) system (*n*-butanol:acetone:methanol:2*N* NH<sub>4</sub>OH) and was used without further purification. [ $^{125}$ I]T3 was purchased from the same commercial source and was purified before use on Sephadex LH-20 columns (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) (15). Purity of the labeled T3 eluted was verified in a

tate; T3, triiodothyronine; T4, thyroxine; TBG, thyroxine-binding globulin; TBPA, thyroxine-binding prealbumin; TETRAC, tetraiodothyroacetic acid; TLC, thin-layer chromatography.

<sup>2</sup> TBG radioimmunoassays performed by Dr. R. P. Levy.

TABLE I  
*Tracer T3 and T4 Distribution among Human Serum Proteins after Polyacrylamide Gel Electrophoresis at pH 9.0*

Serum†	TBG MBC‡	TBPA MBC‡	Binding inhibitor	T3*			T4*		
				TBG	Albu- min	TBPA	TBG	Albu- min	TBPA
N. B.	16	400	Control	46	34	9	43	15	35
			MK-185	22	47	21	19	14	60
			TETRAC	41	39	10	49	21	23
			Salicylate	51	35	7	62	17	15
R. S.	16	189	Control	54	29	9	42	14	36
			MK-185	41	38	12	30	13	52
			TETRAC	45	36	11	53	18	21
			Salicylate	54	32	8	63	19	11
P. D.	21	334	Control	55	29	7	50	14	25
			MK-185	30	38	22	30	9	53
			TETRAC	52	32	5	61	20	9
			Salicylate	66	24	6	70	13	11
D. S.	27	350		68	18	8	44	22	32
G. G.	5	142		10	45	36	24	14	54
J. W.	0	141	Control	3	57	30	2	17	77
			TETRAC	3	62	25	1	19	76
			Salicylate	3	61	26	2	17	74

\* Per cent tracer T3 or T4 in each binding protein zone; tracer unaccounted for in the table was located between TBG and the electrophoretic origin (14) or anodal to TBPA (unbound hormone = less than 2% of tracer) (4).

† N. B., R. S., P. D., sera from euthyroid healthy males; D. S., serum from hypothyroid female, untreated, serum T4 by column = 0.1 µg/100 ml; G. G., J. W., sera from euthyroid healthy males with partial (G. G.) or complete (J. W.) deficiency of serum TBG.

‡ MBC = maximal binding capacity, in µgT4 bound/100 ml serum.

|| Concentrations of binding inhibitors in serum:  $2.9 \times 10^{-3}$  M MK-185;  $3.3 \times 10^{-3}$  M salicylate;  $2.5 \times 10^{-3}$  M TETRAC.

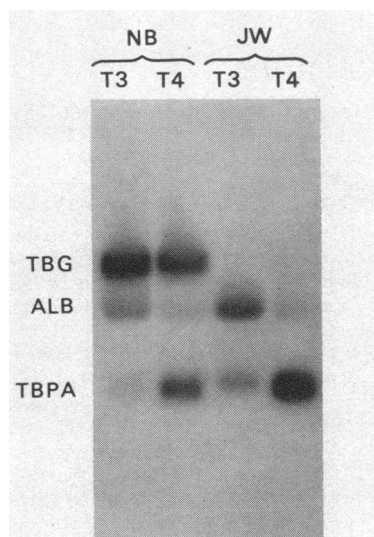


FIGURE 1 Radioautograph of distribution of [<sup>125</sup>I]T3 and [<sup>125</sup>I]T4 in human sera after electrophoresis at pH 9.0. NB, serum from normal subject; JW, serum from subject with total deficiency of TBG. T3 binding is seen in the TBPA zones of both sera, more impressively in the TBG-deficient serum. Anode is at base of figure.

TLC system (above) which satisfactorily resolves T3, T4, and tetraiodothyroacetic acid (TETRAC). Purified material was stored at 4°C in 4% propylene glycol-0.04 N KOH. Unlabeled L-thyroxine sodium (pentahydrate) was obtained from Mann Research Lab. Inc. (New York), unlabeled 3,5,3'-L-triiodothyronine was purchased from Sigma Chemical Co. (St. Louis, Mo.), and 3,5,3',5'-tetraiodothyroacetic acid was obtained from Cyclo Chemical Corp. (Los Angeles, Calif.). Diphenylhydantoin (DPH) sodium was provided by Parke, Davis & Co. (Detroit, Mich.). MK-185 (2-aceto-amidoethyl[4-chlorophenyl][3-trifluoromethyl phenoxy]acetate) was supplied by Merck Sharp & Dohme (West Point, Pa.). Sodium salicylate was reagent grade. Purified human serum TBPA was obtained from Behring Diagnostics, Inc. (Summersville, N. J.). Cow serum was made available by the NIH Animal Center, Poolesville, Md.

*Electrophoretic methods, quantitation of hormone distribution.* Serum to which radioactive T3 or T4 had been added\* was incubated at room temperature for 30 min and then subjected to electrophoresis in polyacrylamide vertical gel slabs at pH 9.0 (4) or pH 7.4 (14). The pH 7.4 system was buffered with PIPES (piperazine-N,N'-bis-[2-ethane sulfonic acid]) (14). The alkaline system consisted of 8% (w/v) polyacrylamide gel, buffered with 0.02 M

\* "Tracer T4" experiments were carried out with less than 0.1 µg [<sup>125</sup>I]T4 added per 100 ml serum; "tracer T3" studies utilized 0.03 µg purified [<sup>125</sup>I]T3/100 ml serum.

**TABLE II**  
*Tracer T3 and T4 Distribution among Proteins in Pooled Human Serum after Polyacrylamide Gel Electrophoresis at pH 7.4: Effects of Binding Inhibitors*

Inhibitor	Concentration of inhibitor <i>M</i>	Control T3 binding*			Control T4 binding*		
		TBG	Albu- min	TBPA	TBG	Albu- min	TBPA
		%	%	%	%	%	%
DPH	$7.9 \times 10^{-3}$	81	188	159	71	137	200
MK-185	$2.9 \times 10^{-3}$	76	180	180	87	175	129
Salicylate	$3.3 \times 10^{-3}$	98	118	91	114	125	65
TETRAC	$2.5 \times 10^{-3}$	96	113	89	121	117	58

\* Control binding (distribution of each radioiodothyronine before addition of inhibitor) = T3: TBG 71%, albumin 12%, TBPA 8%; T4: TBG 63%, albumin 8%, TBPA 17%. Hormone unaccounted for in these distributions was located between TBG and the electrophoretic origin or was unbound (see footnote to Table I). Data presented in this manner indicate, for example, that MK-185 competes with T3 for sites on TBG and displaces T3 to albumin and TBPA. In the presence of MK-185, TBG bound ( $0.76 \times 71$ ) or 54% of tracer T3. Small salicylate and TETRAC effects on the T3-TBPA interaction are suggested by these data; however, the absolute change in amount T3 bound by TBPA in the presence of salicylate or TETRAC at pH 7.4 is comparable to the negligible change at pH 9.0 (Table I).

sodium phosphate, pH 9.0. The slabs were polymerized in a Plexiglas cell (E-C Apparatus Corp., Philadelphia, Pa.) from 2 vol 20% Cyanogum-41 (Fisher Scientific Co., Pittsburgh, Pa.) with 2 vol tetramethylethylenediamine 1% in 0.02 M sodium phosphate, pH 9.0, and 1 vol ammonium persulfate 0.15%. The reservoir buffer was 0.025 M sodium phosphate, pH 9.0, and was circulated during electrophoresis. The conditions of the electrophoresis were 130 v, 100 ma, 5°C for 5 hr. Before and after electrophoresis the pH of the reservoir buffer and of the gel buffer (macrated gel samples) was measured. After electrophoresis the gel buffer pH decreased by 0.4–0.6 U whereas reservoir buffer pH was unchanged. Reservoir buffer was discarded after a single run. After electrophoresis the gels were dried flat (4) and radioautographed for 2–10 days. The gels were then superimposed on radioautographs and zones of T3 or T4 binding were cut out of the gels and counted in a well-type scintillation counter. Radioactivity was also quantitated in zones between the electrophoretic origin and TBG, between albumin and TBPA, and between TBPA and a point 3 cm anodal to TBPA ("unbound hormone") (4). All experiments were performed twice, and data reported are means of determinations of replicate experiments. TBG maximum binding capacities (MBC) and TBPA MBC were measured at T4 concentrations in serum of 100 µg/100 ml and 600 µg/100 ml, respectively, in the pH 9.0 polyacrylamide gel electrophoresis system.

An adjunctive series of electrophoretic experiments was carried out in agarose (General Biochemicals, Div., Chagrin Falls, Ohio), made up 1% in 0.03 M or 0.05 M sodium phosphate buffer, pH 8.6. Experiments were run at 100 v, 120 ma at 5°C for 100 min in vertical gel slabs. There was no alteration in reservoir buffer pH after electrophoresis; gel buffer pH was 8.0–8.2 after each run. After electrophoresis the gels were dried and radioautographed as described for the polyacrylamide gel systems.

**Purified TBPA.** Studies of T3 and T4 binding by purified human TBPA were conducted by adding purified TBPA to cow serum in an amount constituting a TBPA

concentration of 75 mg/100 ml in the serum. Radioactive T3 or T4 was then added to this mixture and to cow serum,

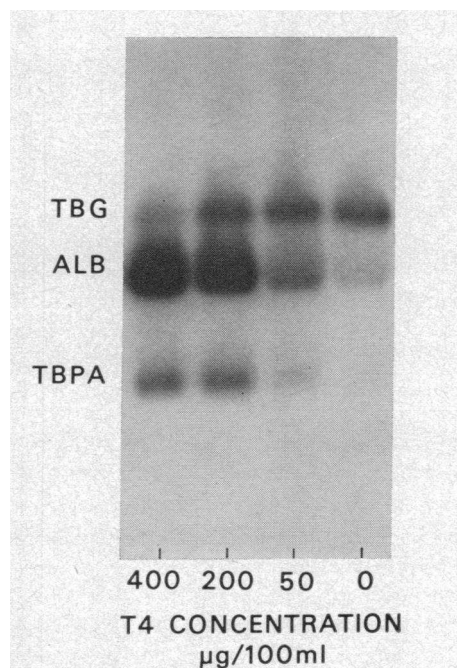


FIGURE 2 Radioautograph of [ $^{125}$ I]T3-containing serum to which progressive additions of unlabeled T4 have been made (electrophoresis carried out at pH 9.0). Redistribution of T3 is seen with increasing serum T4 concentrations, leading to discrete bands of T3 binding in the TBPA zone. Ten µl aliquots were subjected to electrophoresis except for sample without exogenous unlabeled T4 (0 T4) which was run as a 5 µl aliquot. Anode is at base of figure. Complete data from this experiment are plotted in Fig. 3.

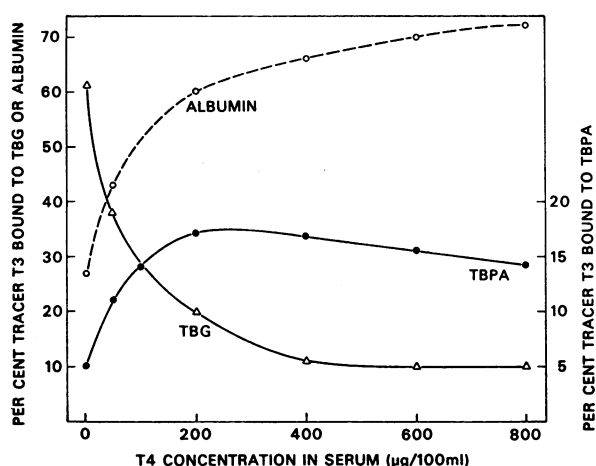


FIGURE 3 Effect of progressive additions of unlabeled T4 on the distribution of [ $^{125}$ I]T3 in pooled sera after electrophoresis at pH 9.0. At T4 concentrations in serum below 200  $\mu\text{g}/100\text{ ml}$ , T4 displaces T3 from TBG to TBPA and albumin. No significant effect of T4 loading on T3-TBPA interaction is seen at T4 levels greater than 200  $\mu\text{g}/100\text{ ml}$ .

alone, and the resultant mixtures subjected to electrophoresis.

## RESULTS

**T3 binding in human serum.** After electrophoresis at pH 9.0 sera from three normal subjects showed 7–9% tracer T3 binding in the TBPA zone (Table I, Fig. 1). In sera from two subjects with TBG deficiency, 30% and 36% of the T3 was located in the TBPA band. In experiments carried out on pooled sera at pH 7.4 (Table II), 8% of the T3 migrated with TBPA. In addition to the data from individual sera and from the pooled sample reported here, we have demonstrated T3 binding by TBPA in five additional normal sera studied at both pH 9.0 and pH 7.4. We have not found any human serum in which TBPA binding of T3 failed to occur.

Serum from P. D. to which radioactive T3 or T4 was added was studied in an agarose gel electrophoresis system. Radioactivity in the tracer T3 experiments varied between 2% and 5% in the TBPA zone in five separate electrophoretic runs; however, there was no discrete band of radioactivity in the TBPA region seen in any of the radioautographs of [ $^{125}$ I]T3-containing serum, in contrast to the tracer T4 studies, the results of which were comparable to agarose gel studies previously reported (16).

**Effects of binding inhibitors on T3 binding in human serum.** DPH and MK-185, inhibitors of T4 binding by TBG (17, 18), displaced T3 from TBG to albumin and TBPA at pH 7.4 (Table II). These data are expressed as *per cent control binding* (4, 19) to facilitate recognition of sites of competition and redistribution of dis-

placed hormone; control binding data, i.e. distribution of radioiodothyronines before addition of inhibitors, are cited in the footnote to Table II.

At pH 9.0 the addition of MK-185 to normal serum caused from 12–22% of tracer T3 to be bound by TBPA (Table I). Salicylate (20) and TETRAC (19) are known to compete with T4 for binding sites on TBPA (actions which are verified in our electrophoretic systems in the tracer T4 data shown in Tables I and II), but neither salicylate nor TETRAC had consequential effects on T3-TBPA interaction. In the case of the TBG-deficient serum studied, salicylate also failed to affect TBPA binding of T4 (J. W., Table I).

**Effects of loading of unlabeled T4 and T3 on [ $^{125}$ I]T3 distribution in serum.** Progressive additions of unlabeled T4 to serum containing [ $^{125}$ I]T3 displaced T3 from TBG to albumin and TBPA (Figs. 2, 3). At T4 concentrations greater than 200  $\mu\text{g}/100\text{ ml}$  there was no further increase in T3 binding in the TBPA zone, and only minimal displacement of T3 from TBPA to albumin. Fig. 2 clearly shows discrete T3 binding in the TBPA zone under conditions of T4 loading.

Additions of unlabeled T3 to the pooled sera containing [ $^{125}$ I]T3 resulted in increasing displacement of the labeled hormone from both TBPA and TBG to albumin; thus, at 800  $\mu\text{g}$  unlabeled T3/100 ml; TBPA binding of [ $^{125}$ I]T3 was reduced by 54% compared with the control sample containing no added unlabeled T3, and TBG binding of [ $^{125}$ I]T3 was decreased by 71%. Additions of unlabeled T3 (up to 800  $\mu\text{g}/100\text{ ml}$ ) to pooled sera containing radioactive T4 did not displace labeled T4 from TBPA.

**Binding of T3 and T4 by purified human serum TBPA.** Electrophoresis of mixtures of purified TBPA and radioactive hormones in buffer resulted in diffuse TBPA and unbound hormone bands. Because cow serum contains no TBPA (2), it was selected as a suitable carrier in which purified TBPA might be subjected to electrophoresis and its ability to bind T3 verified. Fig. 4 shows that [ $^{125}$ I]T3 and [ $^{125}$ I]T4 are bound in discrete bands by TBPA, whereas there is no T3 or T4 binding anodal to cow serum albumin when cow serum has not been enriched with TBPA. Measured radioautographically, the apparent electrophoretic mobility of cow serum albumin was slightly, but consistently, slower in the presence of radioactive T3 than with radioactive T4 (Fig. 4). Stained for protein with 0.1% amido black, however, cow serum containing 2000  $\mu\text{g}/100\text{ ml}$  unlabeled T3 or T4 showed the albumin band to be of precisely the same mobility and width.

## DISCUSSION

Contrary to a number of previous reports, the electrophoretic experiments described here indicate that TBPA

in normal human serum binds a significant fraction of tracer T3 and that in sera which do not contain TBG, or which contain a competitive inhibitor of TBG binding, TBPA may bind large amounts of T3. Confirmatory electrophoretic evidence is supplied by the demonstration of T3 binding by purified human TBPA. These electrophoretic data are in accord with recent studies by Larsen in which binding of T3 by purified TBPA has been shown in ultrafiltration experiments or inferred from ultrafiltration studies of whole serum containing binding inhibitors (21).<sup>4</sup> The addition of unlabeled T4 to serum containing [<sup>125</sup>I]T3 (Figs. 2, 3) displaced T3 from TBG to TBPA and albumin and, interestingly, eventuated in only minimal displacement of T3 from TBPA even when the T4 level in serum was raised to saturation levels for T4 binding by TBPA (600–800 µg T4/100 ml). Progressive loading of serum with unlabeled T3, however, resulted in T3 displacement from TBPA (and from TBG) to albumin.

Displacement studies carried out with additions of salicylate or TETRAC to pooled sera confirmed that these agents competed with T4 for binding sites on TBPA (19, 20), but failed to show interference with TBPA binding of T3. These observations, together with the differential effects of loading of serum with unlabeled T3 or T4, raise the possibility that T3 and T4 interactions with TBPA may not occur at the same site, although other explanations for these findings are possible (for example, existence of heterogeneous species of prealbumins would account for differences in T3 and T4 binding, or T3 interaction with TBPA could have allosteric consequences which prevent competitive T4 interaction with the T3-TBPA complex). The interaction of TBPA and the retinol-binding protein (RBP)-vitamin A complex occurs at a site different from that of T4 binding by TBPA (22). It is apparent from Table I that the expected effect of salicylate on T4-TBPA interaction failed to occur in the TBG-deficient serum which was studied (J. W.). This observation was verified when the initial (replicate) studies were subsequently repeated. The addition of TETRAC to J. W.'s serum also failed to affect T4 binding by TBPA. The intriguing possibility that TBPA in this TBG-deficient serum was functionally distinct from TBPA in normal serum could not be further investigated because of unavailability of additional serum from subject J. W.

It was essential in the present studies that purified T3 be used since commercially available radiotriiodothyronine is often contaminated with radiothyroxine which would misleadingly indicate small amounts of "T3" binding by TBPA. This possibility was excluded in our studies since the [<sup>125</sup>I]T3 used was purified by

<sup>4</sup>Larsen, P. R. Personal communication.

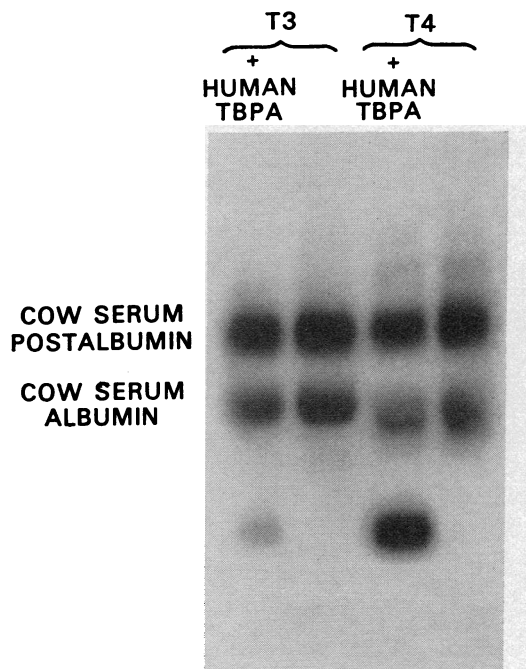


FIGURE 4 Radioautograph of [<sup>125</sup>I]T3 and [<sup>125</sup>I]T4 distribution in cow serum in the absence and presence of purified human TBPA (electrophoresis at pH 9.0). Anode is at base of figure. Cow serum which has not been enriched with human TBPA shows no binding of T3 or T4 anodal to cow serum albumin; TBPA added to cow serum results in both T3 and T4 binding in discrete zones anodal to cow albumin. The possibility that the albumin zone on electrophoresis of cow serum includes a heterogeneous group of proteins, in which T3 and T4 are bound to different species of proteins, is raised by apparent differences in electrophoretic mobility of albumin depending upon which radioiodothyronine is bound. This possibility was supported by protein strains of sera loaded with either unlabeled T3 or unlabeled T4 (see Results) which showed the albumin zones to be of identical electrophoretic mobility.

chromatography on Sephadex LH-20 and shown to contain no T4 by TLC. Moreover, in studies of TBG-deficient sera, and in experiments involving the addition of TBG inhibitors of binding, the amount of radioactivity in the TBPA zone on electrophoresis far exceeded the quantities of T4 which we have found to contaminate commercial [<sup>125</sup>I]T3.<sup>5</sup> In our studies even the use of unpurified T3 would not have compromised experimental interpretation.

Failure of previous electrophoretic systems to demonstrate T3 binding by TBPA is somewhat puzzling. However, Mitchell, Bradford, and Collins (23) have shown that conventional paper electrophoresis results in progressive loss of T3 from albumin binding sites, and it is likely that in various electrophoretic systems com-

<sup>5</sup>There was less than 1% [<sup>125</sup>I]T4 in the unpurified lot of [<sup>125</sup>I]T3 obtained for these studies.

petition between electrophoretic medium and serum proteins for thyroid hormone occurs; in the case of T<sub>3</sub>, media other than polyacrylamide gel may be more effective binders of the hormone than is TBPA. Our own studies of T<sub>3</sub> distribution in serum in another electrophoretic medium (agarose), using phosphate buffer, failed to demonstrate discrete TBPA binding of T<sub>3</sub>. With the experience of others with starch and paper media, the agarose gel results emphasize that polyacrylamide has certain properties which permit the electrophoretic observation of T<sub>3</sub>-TBPA interaction. Differences in electrophoretic buffer, responsible for the initial failure to recognize prealbumin binding of T<sub>4</sub> (1), do not explain previous inability to document T<sub>3</sub> binding by TBPA, since phosphate buffer, used here, has been utilized in other media where T<sub>3</sub> binding by TBPA was not observed.

Previous reports that T<sub>3</sub> may, under certain conditions, migrate in the  $\beta$ -globulin zone on electrophoresis (23, 24), as well as with TBG and albumin, could not be confirmed in our studies. It is also apparent that T<sub>3</sub> distribution among serum proteins does not simply reflect T<sub>4</sub> distribution (although binding proteins have a greater affinity for T<sub>4</sub>), since our T<sub>4</sub>-loading studies showed T<sub>3</sub> to be displaced to TBPA, but only minimally dislodged from TBPA by very high serum T<sub>4</sub> concentrations. In addition, hypothyroxinemic serum (D. S., Table I) showed the same TBPA binding of T<sub>3</sub> as did normal serum.

The physiological significance of T<sub>3</sub> binding by TBPA remains to be elucidated. Despite the binding of small but significant amounts of T<sub>3</sub> by TBPA in normal sera, the presence of TBG, with a higher affinity for T<sub>3</sub> than TBPA, would minimize alterations in T<sub>3</sub>-TBPA interaction (i.e., T<sub>3</sub> displaced from TBPA should be bound by TBG, not remain in the unbound state). Thus, changes in TBPA concentration in the blood should not in normal subjects greatly affect the level of free T<sub>3</sub>. Interference in vivo with T<sub>3</sub> binding by TBPA through the clinical use of inhibitors of TBPA binding also should not affect free T<sub>3</sub> in serum. However, in the TBG-deficient subject, or in the clinical situation in which an inhibitor of TBG binding is present in the blood, alteration in T<sub>3</sub> economy could conceivably derive from changes in TBPA concentration.

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