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Salicylate-induced increases in free triiodothyronine in human serum: *Evidence of inhibition of triiodothyronine binding to thyroxine-binding globulin and thyroxine-binding prealbumin*

P. R. Larsen

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Salicylate-Induced Increases in Free Triiodothyronine in Human Serum

EVIDENCE OF INHIBITION OF TRIIODOTHYRONINE BINDING TO THYROXINE-BINDING GLOBULIN AND THYROXINE-BINDING PREALBUMIN

P. R. LARSEN

From the Division of Endocrinology and Metabolism, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

ABSTRACT Addition of sodium salicylate to human serum at concentrations often obtained during aspirin therapy causes 100–200% increases in free triiodothyronine (T_3) and free thyroxine (T_4) as estimated by ultrafiltration. The increase in free T_3 was unexpected since previous data had suggested that salicylate inhibits binding of T_4 only to thyroxine-binding prealbumin (TBPA) and that T_3 is not bound to this protein. Using ultrafiltration techniques, we demonstrated binding of T_3 to TBPA. The affinity constant for T_3-TBPA binding appears to be slightly greater than that for albumin-T_3 binding. While salicylate inhibits the binding of T_3 (and T_4) to TBPA, it can be predicted that little change will be observed in the free T_3 (or free T_4) without inhibition of thyroid hormone binding to thyroxine-binding globulin (TBG). Using a competitive-binding protein displacement technique, it has been shown that sodium salicylate, like diphenylhydantoin (DPH), inhibits the binding of T_3 and T_4 to TBG. The magnitude of the increase in free T_3 and free T_4 induced by salicylates suggests that interference with TBG binding is its major effect.

Aspirin was administered orally to two normal subjects in quantities sufficient to obtain serum salicylate levels of 20–25 mg/100 ml. This resulted in a decrease of 20–30% in total serum T_3 and T_4 levels. This decrease in T_4 levels is similar in magnitude to that previously observed in subjects receiving DPH. Unlike what has been observed with DPH treatment, therapeutic salicylate levels are associated with increases of 50–75% in the unbound fraction of both T_3 and T_4 which persist throughout an 8–10 day treatment period.

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INTRODUCTION

The recent availability of techniques for measuring triiodothyronine (T_3)[1] in human serum has provoked new interest in this thyroid hormone (1–3). It is speculated that T_3 may provide as much as 50–60% of the body's thyroid hormone requirements (2). Because of the apparent importance of this hormone, we have been interested in the effects of various drugs which might interfere with binding of T_3 to the proteins in human serum leading to subsequent increases in the free form of this hormone.

Extensive prior investigations with thyroxine (T_4) have shown that the small, unbound fraction, free T_4, is the most satisfactory peripheral index of the thyroid status (4). It is postulated that free T_4 is the only, or at least the most readily available, fraction of T_4 present in extracellular fluid that can be utilized by the cells. Similar theoretical arguments are applicable to free T_3. Therefore, agents which interfere with the binding of T_3 to its binding proteins would lead to at least a temporary increase in free T_3. This might be expected to increase the amount of T_3 available to peripheral tissues

[1] *Abbreviations used in this paper:* DPH, diphenylhydantoin; T_3, triiodothyronine; T_4, thyroxine; TBG, thyroxine-binding globulin; TBPA, thyroxine-binding prealbumin; UFT_3 and UFT_4, ultrafiltrable fraction of tracer hormones in serum after labeling with T_3 and T_4, respectively.

and perhaps, because of the rapid onset and high potency of T_3, to cause hypermetabolism.

Previous studies have shown that salicylate and its congeners decrease the binding of T_4 to the binding proteins in human serum (5–7). These drugs were found to interfere specifically with the binding of T_4 to thyroxine-binding prealbumin (TBPA). This conclusion was largely based on the alterations in the distribution of labeled T_4 after paper electrophoresis of human serum. Since T_3 is not thought to be bound to TBPA, we anticipated that addition of salicylate to human serum would cause an increase in free T_4 with no change in free T_3. Administration of this drug might, therefore, offer a way to compare the effects of changes in the free form of only one of the two thyroid hormones. However, preliminary studies showed that addition of sodium salicylate to human serum caused an increase in free T_3 that was nearly as great as the salicylate-induced increase in free T_4.

Simultaneous experiments with serum enriched with T_4 showed that the increased free T_3 induced by salicylates could not be explained by displacement of T_4 from TBPA to TBG. The salicylate effect on free T_3 suggested either that this drug could interfere with TBG-T_3 interaction or that T_3 was, in fact, bound to TBPA, or, perhaps, both. This seemed inconsistent with the previously mentioned conclusions. The following studies were performed to clarify the mechanism of action of salicylate on the protein binding of the two thyroid hormones.

METHODS

Free triiodothyronine and free thyroxine. These values were estimated by determining the ultrafiltrable fraction (UFT_3 and UFT_4) of tracer hormones present after enrichment of human serum with labeled T_3 and T_4 by methods previously described (8). In order to obtain simultaneous measurements, T_3-^{131}I, 0.05-0.20 μg/100 ml, and T_4-^{125}I, 1-3 μg/100 ml serum were added. Both isotopes were obtained from Abbott Laboratories, North Chicago, Ill. The T_4-^{125}I preparations used contained less than 0.3% T_3-^{125}I as a contaminant as determined by paper chromatography in tertiary amyl alcohol-hexane-NH_4OH by methods described previously (9). Overnight dialysis of the 1:1 dilution of T_4-^{125}I with serum removes about 20% of this contaminant as determined by appropriate studies with tracer T_3. Therefore, the over-all contamination of T_4-^{125}I with T_3-^{125}I was less than 0.25%. Since the ratio UFT_3/UFT_4 in pooled serum is about 20:1 the T_3 contaminant in the tracer leads to an artifactual increment in UFT_4 of about 5% which is negligible for the purposes of these studies. Since the salicylate-induced increase in UFT_4 is greater than the increment in UFT_3, the percentage artifactual elevation in the UFT_4 due to contaminating T_3 in the presence of salicylates is less than under base line conditions.

Ultrafiltrate was obtained by centrifugation at 37°C with the pH in whole serum maintained at 7.4–7.6 by an atmosphere of 10% CO_2. The UFT_3 and UFT_4 were corrected for the yield of 89 and 93%, respectively, during the magnesium chloride precipitation step and are expressed as fractions.

Serum T_3 levels were measured in duplicate by a modification of a recently described T_3 immunoassay using unextracted serum and a specific anti-T_3 antibody (10).[2] Normal values for this method in our laboratory are 1.1±0.25 ng/ml (mean±SD).

Thyroxine determinations were performed by the method of Murphy with minor modifications (11). The normal range in our laboratory is 4.5 — 12 μg T_4/100 ml serum. The values are corrected for the 80% yield in the ethanol extraction step. The values for absolute free T_3 and free T_4 were determined by multiplication of the ultrafiltrable fraction by the concentration of total hormone. In the sequential studies of the effects of aspirin administration, all determinations of each parameter were carried out simultaneously to minimize technical variations.

In the studies performed to demonstrate displacement of T_3 and T_4 from TBG, methods identical with those previously described for quantitation of T_3 using TBG were used (9). In this system, 4% human serum in glycine-acetate or barbital buffer, pH 8.6, is enriched with tracer T_3 and T_4. 1-ml aliquots are added to tubes containing various quantities of T_3, T_4, and Na salicylate. The tubes are then allowed to incubate overnight and the bound and free hormones are separated by adsorption of the free tracer hormones to dextran-coated charcoal. The per cent of the tracer remaining in the supernate is expressed as a fraction of the total counts for each isotope.

TBG-binding capacities were estimated by the reverse flow electrophoresis method of Elzinga, Carr, and Beierwaltes and TBPA by the method of Oppenheimer, Martinez, and Bernstein with minor modifications (12, 13).

All solutions were made using distilled, deionized water. Sodium salicylate was obtained from Fisher Scientific Co., Pittsburgh, Pa. Sodium thyroxine pentahydrate, sodium triiodothyronine, crystalline human serum albumin and 5,5-diphenylhydantoin were obtained from Mann Research Labs, Inc., Orangeburg, N. Y. Purified TBPA was obtained from Behring Diagnostics, Inc., Woodbury, N. Y. Statistical analyses were performed using standard methods (14). Tests involving humans were performed with the informed, written consent of the subjects, some of whom were hospitalized on the General Clinical Research Unit. Pooled human serum was obtained through the kindness of the Clinical Chemistry laboratory of the Presbyterian-University Hospital in Pittsburgh.

RESULTS

Effect of in vitro addition of sodium salicylate on UFT_3 and UFT_4. In vitro addition of increasing amounts of sodium salicylate to human serum results in progressive increases in both UFT_3 and UFT_4 (Fig. 1). At a concentration of 60 mg/100 ml there is an increase of 155 and 241% over control values respectively. In this system, addition of 60–80 μg/100 ml of T_4 are required to obtain similar increases in UFT_3 and UFT_4. Therefore, the increase in UFT_3 cannot be attributed to displacement of a small amount of T_4 from TBPA to TBG with a subsequent displacement of T_3. The concentrations of salicylate used are within the therapeutic (20–30

[2] Larsen, P. R. 1971. The direct immunoassay of triiodothyronine in human serum. Submitted for publication.

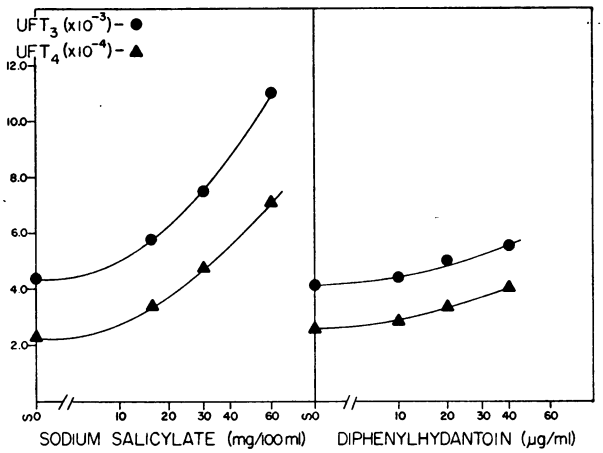

FIGURE 1 Effect of in vitro addition of sodium salicylate or diphenylhydantoin to pooled human serum on the ultrafiltrable fraction of T_3 (UFT_3) and T_4 (UFT_4). The mean of quadruplicate determinations is given. The SEM is too small to be depicted on this scale being $< 3\%$ of the corresponding value in all cases.

mg/100 ml) or toxic (60, or greater, mg/100 ml) ranges observed in man.

For further comparison the effects of the addition of diphenylhydantoin (DPH) are also shown. This agent causes both displacement of T_4 and presumably T_3 from TBG (6). At therapeutic levels (10–20 μg/ml), DPH addition results in considerably less dramatic increases in UFT_3 and UFT_4 though the changes are statistically significant ($P < 0.05$ at 10 μg/ml, $P < 0.01$ at 20 and 40 μg/ml).

Effect of dilution of human serum containing salicylate on UFT_3 and UFT_4. It would be anticipated from the mass law equations that dilution of a weakly bound ligand would decrease its competition with one more firmly bound. For example, dilution of serum containing DPH results in a marked decrease in the apparent effect of this agent on T_4 binding in human serum (15). A similar phenomenon was observed with sodium salicylate (Fig. 2). In these studies, control and salicylate-enriched human sera were progressively diluted as indicated and UFT_3 and UFT_4 determinations made simultaneously. There is a progressive decrease in the apparent effect of salicylate with dilution which is significant for UFT_3 even at a 1:2 dilution. The increase in both UFT_3 and UFT_4, over twofold in the undiluted control specimen, is nearly eliminated at a 1:50 dilution of human serum. These studies underline the necessity for performing studies of weak binding inhibitors in whole serum. In addition, these data demonstrate that the effect of salicylates on the UFT_3 and UFT_4 is reversible.

Comparison of the effects of sodium salicylate and barbital on the binding of T_3 and T_4 in human serum and to human serum albumin. Since the results of previous studies argued against an effect of salicylate either on interference with T_3-TBG binding or T_3-TBPA binding, we initially examined the effect of this agent on albumin-T_3 binding. Both salicylate and the thyroid hormones are known to be bound to this protein. In addition, the effects of barbital, another agent previously demonstrated to interfere with the binding of T_4 to TBPA, were compared with those of salicylate (7). For technical reasons, diluted serum or human serum albumin was used in these studies and sodium salicylate was added to a final concentration of 15 mg/100 ml. In the first entry of Table I, it is seen that both salicylate and barbital cause an increase in the UFT_4. The increase in UFT_4 after dilution of serum in barbital buffer is somewhat greater than that caused by salicylate; however, this may be only a matter of concentration differences. The inhibitory effect of salicylate and barbital on albumin-T_4 binding is considerably greater than that in serum as would be anticipated. The effect of salicylate and barbital on the binding of T_3 in human serum is similar to the effects of these agents on T_4 binding. Thus, both salicylate and barbital displace T_3 from its binding proteins. However, it is clear from the last entry in Table I that the effect of these agents on T_3 binding in whole serum cannot be explained by inhibition of T_3 binding to human serum albumin. The per cent increase in the UFT_3 caused by salicylate and barbital is less with purified human albumin than it is with whole serum. Thus, it

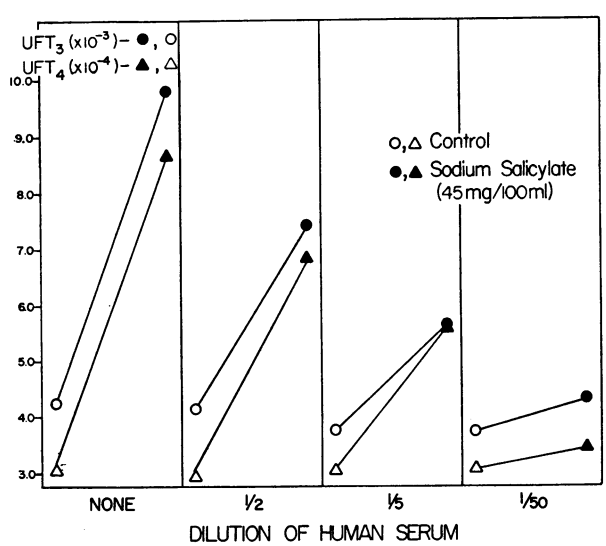

FIGURE 2 Effect of dilution of control and sodium salicylate-enriched samples of human serum in 0.1 M PO_4 buffer, pH 7.4, on the UFT_3 and UFT_4. The salicylate was added and then successive dilutions of this and the control serum were made. Ultrafiltrate was then collected simultaneously from the eight samples. The results are the mean of three experiments.

TABLE I

Effects of Sodium Salicylate and Barbital on the Binding of T_4 and T_3 to Whole Human Serum and to Purified Human Serum Albumin

	PO_4*	Salicylate	Barbital‡
		T_4	
		Serum	
UFT_4 ($\times 10^{-4}$)§	2.3±0.2‖	6.4±0.2	8.7±0.3
Increase, %	—	178	278
		Albumin	
UFT_4($\times 10^{-3}$)	0.9±0.2	4.9±0.2	7.0±0.01
Increase, %	—	445	678
		T_3	
		Serum	
UFT_3($\times 10^{-3}$)	3.0±0.1	6.3±0.3	11.2±0.3
Increase, %	—	110	274
		Albumin	
UFT_3($\times 10^{-3}$)	8.7±0.2	15.3±0.3	19.4±0.1
Increase, %	—	76	123

* Serum or human serum albumin (4% in 0.15 M NaCl) was diluted 1:5 in 0.1 M phosphate buffer, pH 7.8. When indicated, sodium salicylate was added to a final concentration of 15 mg/100 ml.

‡ Serum or human serum albumin (4% in 0.15 M NaCl) was diluted 1:5 in 0.08 M barbital buffer, pH 7.8.

§ Ultrafiltrable tracer T_4 and T_3 are expressed as fractions of the total.

‖ Mean±SEM of quadruplicate samples.

was necessary to examine both the question of salicylate interference with T_3-TBG binding as well as to determine whether or not T_3 was bound to TBPA.

Effects of salicylate on T_3-TBPA binding. Using the ultrafiltration system and simultaneous labeling with T_3-^{131}I and T_4-^{125}I, we found the UFT_3 to be consistently in the range of 0.71 while the UFT_4 was approximately 0.04 in the presence of 1.3×10^{-6} M TBPA (Table II). While T_3 is bound to human TBPA as indicated by the UFT_3 of < 1.0, the binding was considerably less strong than that of T_4. Paper electrophoretic studies of the commercial preparation of TBPA after addition of tracer quantities of T_4-^{125}I did not show any evidence of TBG contamination.

As a control for these studies, similar concentrations of human serum albumin were tested. The binding of T_3 to TBPA seems to be slightly greater than the binding of T_3 to human serum albumin as indicated by the higher UFT_3 in the presence of albumin. It is seen that both the UFT_3 and UFT_4 are increased by salicylate addition to either protein solution indicating this compound interferes with T_3 and T_4 binding to both proteins. The studies in this table were performed in glycine-acetate buffer at pH 8.6. Similar results were obtained in PO_4 buffer at physiological pH, although the binding of T_3 to TBPA appeared somewhat lower in magnitude. The initial UFT_3 was 0.80 in this system.

While these data could explain the salicylate effect, further considerations suggested that this could only be a partial cause. First, while the affinity of T_3 for TBPA is only slightly greater than that of albumin, the concentration of albumin in human serum is at least 200-fold greater than that of TBPA (17). One would anticipate, therefore, at physiological protein concentrations, more T_3 would be bound to albumin than to TBPA. Yet, as previously demonstrated, the effects of salicylate on albumin-T_3 binding were considerably less than they were on the binding of T_3 in whole human serum. Secondly, the affinity of T_3 for TBG is thought to be on the order of 10^9, much greater than the estimated affinity of T_3 for either albumin or TBPA (18). Only about $\frac{1}{3}$ of the binding sites of TBG are occupied by T_4 and T_3 at the concentrations present in normal human serum. Therefore, the small quantities of T_3 (and T_4) displaced from albumin and TBPA by salicylates could be accommodated by TBG with minimal charges in the free hormone fraction. It is, then, difficult to explain how significant elevations in the free T_3 can occur in the presence of salicylates without interference with TBG-T_3 interaction. For these reasons the effect of salicylate on the binding of T_3 and T_4 to TBG was examined.

Effects of sodium salicylate on T_3 and T_4 binding to TBG. The effects of salicylate on TBG binding were examined in an assay system similar to that used in the competitive-binding displacement assay used for T_3 determinations (9). This was less cumbersome than the ultrafiltration system, and provided similar qualitative information. Initially, whole human serum was diluted 1:25 in barbital buffer to minimize TBPA and albumin binding of tracer T_3 and T_4 but similar results have been obtained in both glycine-acetate buffer, pH 8.6, and phosphate buffer, pH 7.4. After addition of various

TABLE II

Inhibition of T_3 and T_4 Binding to TBPA and Human Serum Albumin by Sodium Salicylate

	Control		Salicylate (15 mg/100 ml)	
	UFT_3*	UFT_4*	UFT_3	UFT_4
TBPA (1.3×10^{-6} M)§	0.71±0.01‡	0.040±0.006	0.90±0.02	0.14±0.01
Albumin (1.2×10^{-6} M)	0.80±0.03	0.36±0.01	0.90±0.03	0.57±0.01

* Ultrafiltrable tracer T_3 and T_4 are expressed as fractions of the total.

‡ Mean±SEM of quadruplicate determinations.

§ Calculated using molecular weight of 50,000 (16).

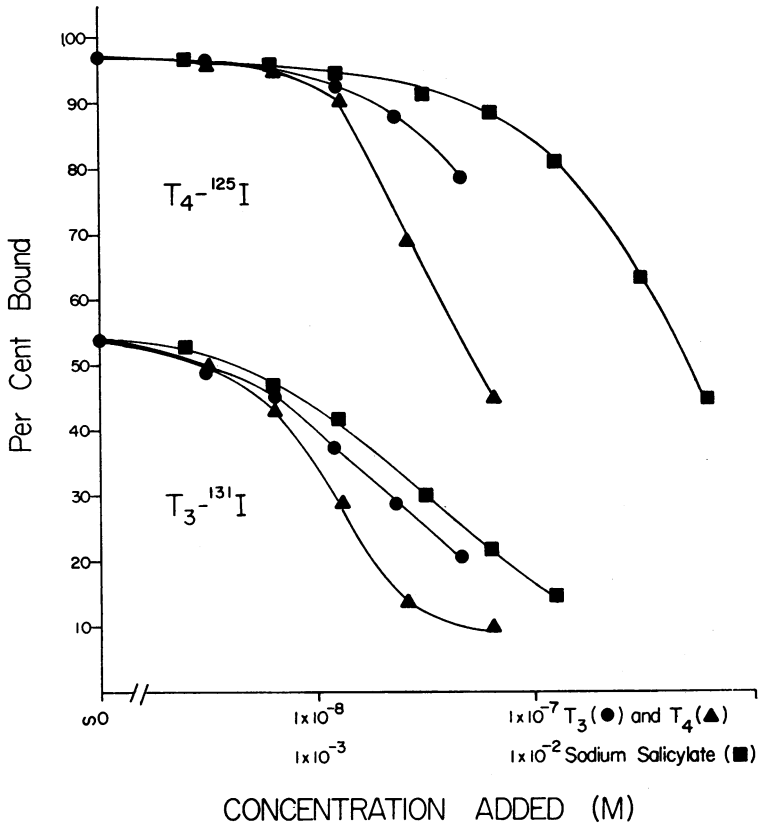

FIGURE 3 Displacement of T_4-^{125}I and T_3-^{131}I from the binding proteins in human serum diluted 1:25 in barbital buffer 0.08 M, pH 8.6. Bound and free tracer hormone was separated by adsorption of unbound hormone to dextran-coated charcoal. The mean of triplicate determinations is given, the SEM is $< 1.5\%$ for all points. The concentration of added binding inhibitor is given as moles/liter.

amounts of T_3,T_4 or sodium salicylate, 1-ml samples of the mixture were equilibrated overnight and then bound and free hormones were separated by addition of dextran-coated charcoal. As is seen in Fig. 3, both T_3 and T_4 displace the labeled iodothyronines from the binding proteins present in human serum. In addition, sodium salicylate readily displaces T_3 and, at higher concentrations, T_4 from these binding proteins.

While physicochemical considerations suggest that TBG is the only binding protein present in human serum with a sufficiently high affinity for T_3 and T_4 to act as a competitive binding protein, it was necessary to prove this conclusively. It has been demonstrated by Pensky and Marshall that TBG can be isolated from human serum using affinity chromatography with T_4 coupled to Sepharose (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) (19). Applying the same principle, T_4-Sepharose, prepared as described by these authors, was added to human serum and incubated at 4°C overnight with stirring. Comparison of paper electrophoretic patterns after addition of T_4-^{125}I in tracer amounts showed that TBG binding was almost eliminated in serum treated in this way (Fig. 4). If the small deflections in the TBG

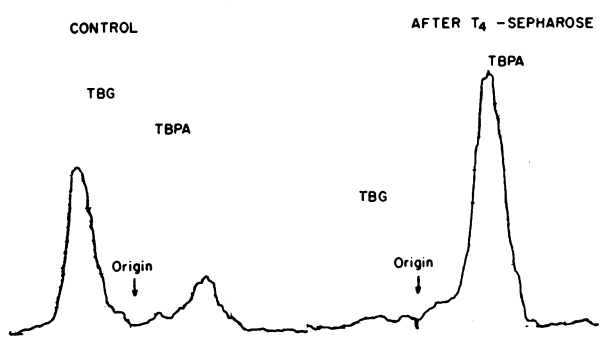

FIGURE 4 Distribution of tracer T_4 in human serum before and after TBG adsorption with T_4-Sepharose. Reverse flow electrophoresis of serum was performed in glycine-acetate buffer pH 8.6 after addition of tracer quantities of T_4-^{125}I (< 2 μg/100 ml).

area of the T_4-Sepharose adsorbed sera are due to TBG-bound T_4, by planimetry, it represents less than 8% of the original TBG-binding capacity.

TBPA-binding capacity in serum was unchanged by T_4-Sepharose adsorption and the T_4 concentration increased only 2 μg/100 ml. The latter occurred due to the fact that even with repeated attempts to purify the T_4-Sepharose, small quantities of presumably noncovalently bound T_4 adhere to the material and are subsequently bound to the serum proteins. It is obvious that enrichment of serum with large quantities of T_4, such as might occur with unwashed T_4-Sepharose preparations, could lead to a similar decrease in the percentage of tracer bound to TBG that is shown in Fig. 4 (though one would expect to see much greater binding in the albumin area under these conditions).

Since the same serum could be compared before and after TBG elimination, its role in this assay system could be clarified. Table III shows comparison of T_3 and T_4 binding of these sera in both glycine-acetate and barbital buffer in the same system described for Fig. 3. It is apparent that the binding of T_3 is inhibited by barbital. There is also slight inhibition of T_4 binding in barbital buffer. After removal of TBG, the per cent T_3 bound falls to a level of 13.3 in glycine acetate and 6.5 in barbital buffer demonstrating that TBG is the critical T_3-binding protein in this system. While there is a significant decrease in the per cent T_4 bound after removal of TBG, it is only after inhibition of TBPA and albumin binding in barbital buffer that the importance of TBG-T_4 binding in this system is observed. The control data demonstrate that there is no significant difference in

TABLE III

Binding of T_3-^{131}I and T_4-^{125}I in Dilute Human Serum before and after Removal of Thyroxine Binding Globulin (TBG) as Assessed by Dextran-Coated Charcoal Separation of Bound and Free Tracer

	Buffer*	Amount bound to serum proteins	
		T_3-^{131}I	T_4-^{125}I
		%	%
Control	Glycine acetate	70.2±0.7§	96.4±0.6
	Barbital	43.3±0.7	94.6±0.3
After removal of TBG	Glycine acetate	13.3±0.3	82.6±0.6
	Barbital	6.5±0.4	22.6±0.7
Buffer control (No serum)	Glycine acetate	2.7±0.3	3.1±0.7
	Barbital	3.4±0.3	3.7±0.1

* pH 8.6.

‡ Mean±SEM of triplicate determinations in two experiments.

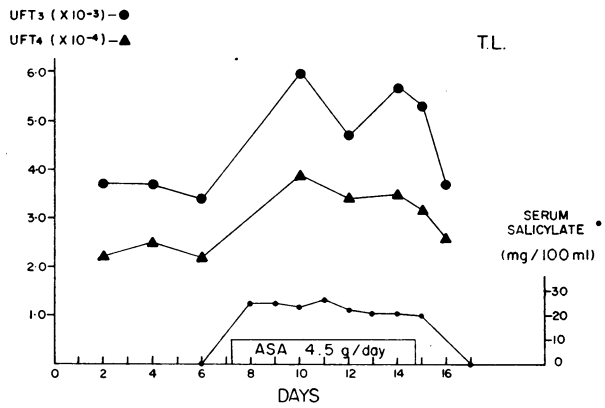

FIGURE 5 UFT_3 and UFT_4 in a subject receiving chronic aspirin therapy. Values given are single determinations which were estimated simultaneously using the same tracer.

the two systems with respect to the binding of free tracer to the dextran-charcoal in the absence of serum. The data in Table III show that in this system (the same as that of Fig. 3), a maximum of about 6.5% of the tracer T_3-^{131}I is bound to TBPA and albumin and likewise about 22.6% of the tracer T_4 is bound to these two proteins under base line conditions. Therefore, any decrease in the percentage of tracer T_3 bound greater than 6.5% and of T_4 greater than 22.6% implies interference with TBG binding of these two hormones. Since sodium salicylate at 6×10^{-3} M causes a net displacement of about 41% of the T_3-^{131}I and at 6×10^{-2} M displaces about 52% of the T_4-^{125}I (Fig. 3), one may conclude that, in this system, salicylate inhibits the binding of T_3 and T_4 to TBG.

Effect of salicylate on T_3 and T_4 binding in vivo. While previous studies have shown decreases in the PBI in patients treated with salicylates, no reports of ultrafiltrable T_3 or T_4 have appeared (20). If salicylates interfere with TBG-T_4 and T_3 binding one would anticipate a decrease in the total T_3 and T_4 and increases in the UFT_3 and UFT_4. We have studied the effects of aspirin administration in two subjects under controlled conditions. Three base line determinations were obtained during a 6 day control period. Specimens during aspirin administration were obtained every other day over a subsequent 8 to 10 day period. There was an immediate and persistent increase in the UFT_3 and UFT_4 in both subjects. Fig. 5 shows the relationship of these changes to the salicylate levels in one of these. Maintenance of a salicylate concentration on the order of 20–25 mg/100 ml was associated with increases in the free fraction comparable to those obtained in vitro at this concentration. Unlike what has been observed during DPH therapy, this increase in the free fraction of both T_3 and T_4 appears to be maintained (8, 15).

TABLE IV

Effect of Aspirin Administration on Free Triiodothyronine (FT_3) and Free Thyroxine (FT_4)

	Subject T. L.						Subject R. R.					
	T_3	UFT_3	FT_3	T_4	UFT_4	FT_4	T_3	UFT_3	FT_3	T_4	UFT_4	FT_4
	ng/ml	$\times 10^{-3}$	*ng/100 ml*	*μg/100 ml*	$\times 10^{-4}$	*ng/100 ml*	*ng/ml*	$\times 10^{-3}$	*ng/100 ml*	*μg/100 ml*	$\times 10^{-4}$	*ng/100 ml*
Control period												
1	1.05	3.7	0.39	7.7	2.2	1.7	1.13	4.6	0.52	9.0	3.0	2.7
2	0.80	3.7	0.30	7.7	2.5	1.9	0.98	3.7	0.36	8.9	2.6	2.3
3	0.80	3.4	0.27	7.3	2.2	1.6	0.93	4.8	0.45	8.5	2.9	2.4
Mean ±SEM	0.88±0.08	3.6±0.1	0.32±0.04	7.6±0.1	2.3±0.1	1.7±0.1	1.01±0.06	4.4±0.3	0.44±0.05	8.8±0.2	2.8±0.1	2.5±0.1
Aspirin period												
4	0.70	6.0	0.42	5.9	3.9	2.2	0.85	9.8	0.83	6.3	5.3	3.3
5	0.70	4.7	0.33	5.6	3.4	1.9	0.70	5.9	0.41	6.5	4.4	2.9
6	0.62	5.7	0.35	4.4	3.5	1.5	0.76	7.8	0.59	7.0	5.3	3.7
7	0.78	5.3	0.41	5.8	3.2	1.9	0.58	6.4	0.37	—	4.4	—
Mean ±SEM	0.70±0.03	5.4±0.3	0.38±0.02	5.4±0.4	3.5±0.1	1.9±0.1	0.72±0.06	7.5±0.9	0.55±0.10	6.6±0.2	4.9±0.3	3.3±0.2
$\frac{\text{Treatment}}{\text{Control}}$, %	80	150	119	71	152	112	71	170	125	75	175	132
*P**	<0.05	<0.01	NS	<0.01	<0.01	NS	<0.02	<0.05	NS	<0.01	<0.01	<0.05

* *P* determined by *t* test for unpaired samples.

In Table IV are shown the effects of aspirin administration on the absolute free T_3 and free T_4 in the two subjects. While it is difficult to know whether it is accurate to average the samples during the entire treatment period due to disequilibrium conditions, this has been done for the purposes of these initial studies. With regard to T_3 levels, there is a suggestion of a decrease in the total amount of T_3 to about 76% of control in both subjects. This compensates to some extent for the increased UFT_3 resulting in an average increase in free T_3 of only about 23% in these subjects. The decrease in T_4 is substantial, about 25–30%, similar to the effect of DPH therapy (8, 15, 21). Again this compensation results in only a slight increase in the absolute free T_4. These two studies were carried out for a period of about 8 to 10 days of salicylate administration. Longer periods of aspirin treatment will be necessary to determine whether this slightly elevated free hormone concentration persists under steady-state conditions.

DISCUSSION

The above data indicate that sodium salicylate interferes with T_3 and T_4 binding to TBG. This interference was first demonstrated in human serum by Osorio in 1962 (22). In these studies, paper electrophoresis was used but subsequent studies using this technique have emphasized only the salicylate effect on TBPA-T_4 binding (7).

Apparently, the effect of salicylates on the distribution of labeled hormones during paper electrophoresis is markedly greater on TBPA than it is on TBG. Thus, a different system is desirable to demonstrate the magnitude of the inhibition of TBG binding of T_3 and T_4. This could conceivably be done in paper electrophoretic studies with serum completely devoid of TBPA.

While surprising in light of current concepts, one could predict the interference of salicylates with T_3 and T_4 binding to TBG from theoretical considerations based on recently available data. Woeber and Ingbar have shown that complete elimination of TBPA by immunoadsorption increases the free T_4 only an average of 22% (23). Yet at a concentration of 60 mg salicylate/100 ml, the UFT_4 in our system is increased 241%. Therefore, salicylate inhibition of TBPA binding is not playing a major role at this salicylate level. In addition, using assumptions based on the data of these and other investigators regarding T_4 distribution and current estimates of affinity constants for the various binding proteins, one can calculate the theoretical effect of complete elimination of TBPA and albumin binding using the mass action equation developed by Robbins and Rall (reference 4, see Appendix). Using this formula, one estimates that after complete inhibition of T_4 binding to both albumin and TBPA, there will be only a 30% increase in the free T_4. This emphasizes the importance of TBG-binding in the determination of the amount of T_4 that is free. Thus, at higher salicylate levels, interference with TBG-T_4 binding must be a factor and it is presumably present at lower concentrations to some extent as well. Since the affinity of T_3 for all the binding proteins is considerably less than that of T_4, T_3-TBG interaction will be inhibited at lower salicylate levels.

The per cent increase in free T_4 due to salicylate addition is moderately greater than that in free T_3 (241% vs. 155% at 60 mg/100 ml salicylate). However, the fraction of bound T_3 displaced by salicylate is greater than that of T_4. This would be anticipated from the data in Fig. 3 and is a result of the lower binding affinity of T_3 vs. T_4 for TBG. This can be quantitated by comparing the net increase in the UFT_3 in the presence of 60

mg/100 ml salicylate (approximately 6.7×10^{-3}) with the net increase in the UFT$_4$ (approximately 5.3×10^{-4}). The ratio of the two fractional increases is about 13:1, UFT$_3$:UFT$_4$. Nevertheless, the original fraction of T$_3$ free is about 20-fold greater than the fraction of T$_4$ free (4.3×10^{-3} vs. 2.2×10^{-4}, Fig. 1). Therefore, sodium salicylate does cause a disproportionate increase in UFT$_4$ compared with the base line relationships. This could be a result of salicylate interference with T$_4$-TBPA binding which is probably more quantitatively significant than T$_3$-TBPA binding. It is postulated that T$_3$ and T$_4$ are bound to the same site on TBG (18). In a simple system containing a single protein with one binding site, the free T$_4$:T$_3$ ratio should remain constant in the presence of a competitive binding inhibitor.

The at least temporary increases in free T$_3$ and free T$_4$ in human subjects during 1 wk of aspirin therapy is of considerable interest both clinically and theoretically. For many years the similarity of the effects of salicylate and thyroid hormones on oxygen consumption have been recognized (24). This similarity of action extends even to the demonstration in rats that salicylate will, at least temporarily, suppress TSH (25). If free hormone is an index of the quantity of thyroid hormone available to the cells, then elevation of free T$_4$, and particularly free T$_3$ with its more rapid action and greater potency, may lead to symptoms of hyperthyroidism. While it is recognized that salicylates have T$_4$-like effects on subcellular organelles, e.g. to uncouple oxidative phophorylation in mitochondria, the possibility that increases in the utilization of T$_3$ and T$_4$ due to unbinding of these hormones from their binding proteins also occurs during salicylate therapy cannot be excluded (26). The most likely situation in which this could occur would be during salicylate intoxication in children where levels of 50-100 mg/100 ml salicylate are commonly seen. We are currently evaluating the possible significance of drug-induced hyperthyroidism in these patients. It is to be emphasized that at new steady-state concentrations of salicylate, T$_3$, and T$_4$, a return to pretreatment levels of absolute free T$_3$ and free T$_4$ concentrations would be anticipated. As pointed out previously under Results, the small increase in absolute free hormone over the 1st wk of therapy may only be temporary. Only if salicylates affect some other, perhaps cellular, binding site, could a persistent increase in free hormone occur. If only extracellular binding proteins are affected, one would anticipate that the increases in UFT$_3$ and UFT$_4$ would be completely balanced by decreases in total T$_3$ and T$_4$. There is evidence that this is occurring in these two subjects and that only more time would be required for complete equilibration.

The inability of previous investigators to demonstrate T$_3$ binding to TBPA is not surprising in that studies of this hormone using various electrophoretic techniques are complicated by the relatively low affinity of T$_3$ for the binding protein compared with its affinity for the supporting media. Purified TBPA has not been studied for T$_3$ binding in ultrafiltration or equilibrium dialysis systems. It would appear from these preliminary data that the binding of T$_3$ to TBPA is slightly stronger than that of T$_3$ to albumin. The affinity constant for T$_3$-albumin interactions is on the order of 3×10^5 (18). However, the approximately 200-fold concentration excess of albumin relative to TBPA would suggest that more T$_3$ would be transported bound to this protein than to TBPA. Further modifications in electrophoretic techniques will be required to evaluate this question. However, in the patient with TBG deficiency, TBPA should be an important T$_3$ carrier.

While there are various other theoretical aspects of these data, we have recently been able to apply these observations to a practical problem. In the direct immunoassay of T$_3$ in human serum, there is considerable competition for T$_3$ between the antibody and the TBG present in human serum. While this is more important in systems in which bound and free tracer T$_3$ are separated by adsorption of the free T$_3$ to various agents, the presence of TBG is also theoretically disadvantageous in the double-antibody system. We have found that salicylate will block the binding of 90 to 95% of T$_3$ to TBG and this agent currently is being used in our direct immunoassay for T$_3$ (10). It also has the advantage of very low cross-reactivity with the T$_3$ antibody, is readily available and water soluble.

In summary, it would appear that, as might have been predicted from theoretical considerations, salicylate interferes with the binding of T$_3$ and T$_4$ to both TBPA and TBG. The increase in available free T$_3$ and free T$_4$ during the initial phases of aspirin administration may contribute to hypermetabolism associated with the administration of this agent. High concentrations of sodium salicylate can also be used to block the binding of T$_3$ to TBG during the direct immunoassay of T$_3$ in human serum.

APPENDIX

The expression describing the concentration of free T$_4$ originally formulated by Robbins and Rall is as follows (4):

$$(T_4) = \frac{(T_4 \cdot TBG) + (T_4 \cdot TBPA) + (T_4 \cdot ALB)}{[K_{TBG}(TBG)] + [K_{TBPA}(TBPA)] + [K_{ALB}(ALB)]}$$

where (T_4) = free T$_4$, K_{TBG}, K_{TBPA}, K_{ALB} are the binding affinity constants for the respective proteins, (TBG), (TBPA), and (ALB) are the concentrations of unoccupied binding sites on the three binding proteins and $T_4 \cdot TBG$, $T_4 \cdot TBPA$ and $T_4 \cdot ALB$ are the concentrations of T$_4$ bound to the respective binding proteins. All concentrations are moles/liter. The following affinity constants, binding capacities, and distribution of T$_4$ are assumptions based on previously published data

using a T_4 concentration of 7.7 μg/100 ml (16, 18, 23). In addition, it is assumed that there is one primary binding site/mole of binding protein.

	K	T_4 binding capacity	T_4 bound
		moles/liter	*moles/liter*
TBG	1.6×10^{10}	26×10^{-8}	7.5×10^{-8}
TBPA	1.3×10^{7}	320×10^{-8}	1.5×10^{-8}
ALB	6.2×10^{5}	5.7×10^{-4}	1.0×10^{-8}
			Total $T_4 = 10 \times 10^{-8}$

Substitution in the above equation gives the following:

$$(T_4) = \frac{10 \times 10^{-8}}{[(1.6 \times 10^{10})(18.5 \times 10^{-8})] + [(1.3 \times 10^{7})(318 \times 10^{-8})] + [(6.2 \times 10^{5})(5.7 \times 10^{-4})]}$$

and

$$(T_4) = 3.0 \times 10^{-11}$$

and

$$UFT_4 = \frac{(T_4)}{\text{Total } T_4} = \frac{3.0 \times 10^{-11}}{10 \times 10^{-8}} = 3.0 \times 10^{-4}.$$

After complete inhibition of T_4 binding to TBPA and albumin, the following conditions obtain in vitro:

$$(T_4) = \frac{10 \times 10^{-8}\ \text{M}}{(1.6 \times 10^{10})(16 \times 10^{-8})}$$

and

$$(T_4) = 3.9 \times 10^{-11}$$

and

$$UFT_4 = \frac{3.9 \times 10^{-11}}{10 \times 10^{-8}} = 3.9 \times 10^{-4}.$$

Therefore, the relative increase in UFT_4 predicted based on current estimates of the binding affinities and protein concentrations can be calculated:

$$\frac{3.9 \times 10^{-4}}{3.0 \times 10^{-4}} \times 100 = 130\% \text{ of original value.}$$

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