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The Effects of Noncalorigenic Congeners of Salicylate on the Peripheral Metabolism of Thyroxine *

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Various experiments have indicated the presence in whole human serum of three major thyroxine (T_4)-binding [1] proteins: T_4-binding globulin (TBG), T_4-binding prealbumin (TBPA), and albumin (1, 2). It is well established that, *in vitro,* TBG and TBPA are the major T_4-binding proteins, whereas albumin is a relatively weak secondary carrier (3–5). These proteins interact with the hormone in a reversible binding equilibrium in which the majority of the hormone is bound but a measurable proportion is unbound or free. Free T_4 has been suggested as the metabolically active component available to the cells *in vivo* for degradation, excretion, and initiation of hormonal action, with the bound hormone acting as a metabolically inert reservoir (3–5).

The role of TBG in the transport and peripheral metabolism of T_4 in man is well accepted. This acceptance is based on studies demonstrating a consistent relationship between changes in the T_4-binding activity of TBG, as assessed by electrophoresis *in vitro* and changes in the concentration and fractional turnover of T_4 *in vivo* (6–8). From these observations, the conclusion has been drawn that TBG regulates the peripheral metabolism of T_4 by limiting the concentration of unbound hormone.

The existence of TBPA as a normal constituent of human serum has been satisfactorily demonstrated by several techniques, and earlier doubts concerning its function in binding T_4 at physiological pH have been dispelled (5, 9, 10). The role, if any, of TBPA in regulating the peripheral metabolism of T_4 *in vivo* has remained uncertain. As in the case of TBG, attempts have been made to correlate changes in the T_4-binding activity of TBPA *in vitro* with changes in the volume of distribution and fractional turnover of hormone *in vivo.* Thus, certain abnormal states, such as fever and malignancy, have been found accompanied by a decrease in T_4 binding by TBPA (4, 10, 11) and an increase in the fractional rate of turnover of T_4 (12, 13). Since these states are usually associated with hypermetabolism, and since in the absence of known alterations of T_4 binding, hypermetabolism per se may be accompanied by an increase in the fractional turnover of hormone (12, 14), changes in T_4-metabolism in these states cannot be ascribed to changes in hormonal binding. Similarly, salicylate and 2,4-dinitrophenol have been shown to decrease T_4 binding by TBPA *in vitro* (5, 15, 16) and to accelerate the fractional turnover of T_4 in man (17, 18), but since both agents uncouple oxidative phosphorylation and induce a severe hypermetabolism (19, 20), again the changes in T_4 metabolism that these agents induce cannot necessarily be ascribed to an inhibition of T_4-binding by TBPA.

Previous reports have indicated that certain congeners of salicylate do not uncouple oxidative phosphorylations *in vitro* (19) or stimulate oxygen consumption *in vivo* (21, 22). We therefore made a search among these compounds for inhibitors of T_4 binding by TBPA in order to study their effects on T_4-metabolism in man.

Methods and Materials

In vitro studies

The effect of benzoic acid and a number of its hydroxy-, dihydroxy-, and amino-substituted derivatives [2] on the binding of T_4 in normal serum was assessed by electrophoretic and dialysis techniques.

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[1] T_4 refers to the levorotatory isomer of thyroxine.

[2] Obtained from K & K Laboratories, Jamaica, N. Y.

Assessment of T_4 binding by filter paper electrophoresis. By methods described in detail elsewhere (8), electrophoretic studies of the binding of T_4 were carried out in fresh serum from two normal donors. Samples of serum were enriched with a tracer amount of I^{131}-labeled T_4[3] (2 μc per ml, approximately 4 μg per 100 ml) and varying quantities of stable T_4, sufficient to permit estimation of the T_4-binding capacities of TBG and TBPA. Inhibitory effects of benzoic acid derivatives were tested in two ways. First, serum was enriched with derivatives to the desired molarity by adding to nine parts of serum one part of the derivative dissolved in isotonic saline solution; control sera were comparably diluted with saline alone. Second, derivatives were tested for inhibitory potency by adding them directly to the buffer in which electrophoresis was performed. The pH of the buffer was readjusted when necessary.

Electrophoresis of serum specimens was performed on Whatman 3 filter paper in Tris-maleate buffer, pH 8.6 (8). In experiments with derivatives added directly to serum, control and experimental samples were subjected to electrophoresis on the same filter paper sheet. In experiments in which buffer media were enriched with derivatives, duplicate samples of labeled serum were concurrently subjected to electrophoresis in unenriched buffer. Electrophoresis was allowed to proceed for 15 hours at 120 v. Assessment of the distribution of labeled T_4 among TBG, TBPA, and albumin and calculation of the T_4-binding capacities of TBG and TBPA were carried out by methods described in detail elsewhere (8). All experiments were performed at least twice and usually more often; results obtained with each derivative in separate experiments agreed closely.

Assessment of T_4 binding by agar gel electrophoresis. Studies were conducted on glass slides in an agar gel[4] (100 mg agar per 100 ml) prepared in 0.10 M phosphate buffer, pH 7.4.[5] Sera were enriched only with tracer quantities of I^{131}-labeled T_4. Samples of normal serum were diluted (9:1) with benzoic acid derivatives dissolved in isotonic saline solution. Control specimens were comparably diluted with saline alone. Electrophoresis was allowed to proceed for 3 hours at a current of 10 ma. Specimens were then dried under an infrared lamp, and radioautographs were prepared according to methods described previously (5). Thereafter, with the aid of the radioautographs, the zones containing TBG, albumin, and TBPA were sectioned, removed from the slide, and counted directly in a well-type scintillation counter.

Assessment of T_4 binding by dialysis techniques. The effect of benzoic acid derivatives on the rate of dialysis of T_4 from normal human serum was determined by methods, detailed elsewhere (5), for assessing the effects of salicylate itself.

In vitro studies of thyroxine degradation. Slices of kidney cortex were obtained from freshly killed Sprague-Dawley rats. Approximately 100 mg of tissue was placed in Erlenmeyer flasks containing 2.0 ml of Krebs-Ringer phosphate buffer, pH 7.4, enriched with either labeled T_4 alone (4 μc per ml, approximately 8 μg per 100 ml) or labeled T_4 and benzoic acid derivative. Slices from each rat were apportioned into duplicate control and experimental vessels. These, together with vessels containing no slices and serving as tissue-free controls, were then incubated under 100% oxygen at 37° C for 3 hours. Thereafter, 1 ml of human serum was added to the incubation mixture to stop further hormonal degradation and to prevent the subsequent artifactual appearance of iodide during chromatography (23). Tissues were then homogenized in their own serum-enriched incubation media, and samples of the homogenate were subjected to ascending filter paper chromatography in a butanol-dioxane-2 N ammonia (4:1:5) solvent system. Localization of the substrate T_4 and the products of hormonal degradation and determination of their relative proportions were carried out with an automatic strip scanner and electronic integrator by methods described previously (24). Values for the percentile degradation of T_4 were corrected for the non-T_4 radioactivity present in the tissue-free control samples.

In vivo studies

Studies of thyroxine turnover in vivo. Studies of the turnover and degradation of I^{131}-labeled T_4 were carried out in eight patients: four of the patients had no endocrine or systemic disease; three had primary myxedema, one (A.S.) receiving treatment with 3 g of desiccated thyroid daily, and the other two (M.F. and B.P.) being untreated. The eighth patient, who had hypopituitarism, was studied twice before and once during replacement therapy with cortisone. *In vivo* metabolism of T_4 was assessed by methods detailed elsewhere (25). Briefly, each patient was given a single iv injection of 50 μc (approximately 1.5 μg) of I^{131}-labeled T_4 dissolved in 1% human serum albumin. In patients with normal thyroid function, thyroidal recycling of inorganic I^{131} liberated by the peripheral degradation of labeled hormone was prevented by the administration of 30 mg of methimazole every 6 hours. After injection of the labeled hormone, daily blood samples and, in patients R.G. and B.P., 24-hour urine collections were obtained for a total of from 14 to 21 days. After an initial control period of 6 to 8 days, gentisic acid, gamma-resorcylic acid, or, in the case of M.M., salicylamide was given in gelatin capsules in divided daily doses of from 4 to 12 g. The derivative was continued for from 6 to 9 days. In most instances, samples of blood were obtained for several days after the derivative had been discontinued. In two patients, A.S. and B.P., para-hydroxybenzoic acid was begun when gamma-resorcylic acid was stopped, and collection of blood samples was continued for an additional 6 to 7 days.

The fractional rate of hormonal turnover (k) during the control and experimental periods was determined from the declining concentration of the radioactivity in

[3] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

[4] Difco Laboratories, Detroit, Mich.

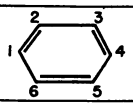
[5] The authors are grateful to Dr. Frederick S. Bigelow for assistance in performing this technique.

serum by the method of least squares. Data obtained during the first 24 to 48 hours of the control and experimental periods, however, were omitted from this calculation to permit the initial equilibration or re-equilibration, respectively, of the injected labeled material. Values for the T_4 distribution space (TDS) during the initial control period were calculated as the quotient of the injected radioactivity and the zero-time value of the radioactivity in serum obtained by extrapolating the calculated regression equation. The TDS during the administration of the benzoic acid derivatives, however, could not be calculated by this method. Accordingly, the percentage of the injected radioactivity remaining in the body at the beginning of the experimental period was calculated from the control fractional turnover rate. The TDS during the experimental period could then be calculated as the quotient of this quantity and the simultaneous concentration of the radioactivity in serum obtained from the calculated regression equation for the experimental period.

The daily clearance of T_4 and the daily rate of disposal of hormonal iodine were calculated for each period from the calculated values for k and TDS and the concentration of hormonal iodine in the serum (4). In two patients, R.G. and B.P., the clearance of T_4 by degradative, rather than excretory, processes was calculated before, during, and after administration of the benzoic acid derivative (8). This "degradative clearance" was calculated from the daily 24-hour urinary excretion of I^{131} and the mean concentration of the radioactivity in serum during that same 24-hour period.

Either serum protein-bound iodine (PBI) or butanol-extractable iodine (BEI)[6] along with basal oxygen con-

[6] Analyses for PBI and BEI were performed in the Boston Medical Laboratory by a modification of the method of Zak (26).



SUBSTITUTIONS FOR HYDROGEN ATOMS						NAME OF COMPOUND	ADDITION TO SERUM: ENDOGENOUS T4-TBPA % Control		ADDITION TO BUFFER 30 mg/100 mL (±2×10⁻³M): TBPA BINDING CAPACITY % Control
1	2	3	4	5	6		1×10⁻³M	2×10⁻³M	
COOH	-	-	-	-	-	Benzoic Acid	95.2	91.4	96.7
COOH	OH	-	-	-	-	Salicylic Acid	83.7	62.5	49.4
COOH	-	OH	-	-	-	m-Hydroxybenzoic Acid	102.9	92.8	95.1
COOH	-	-	OH	-	-	p-Hydroxybenzoic Acid	100.0	99.6	107.8
COOH	OH	OH	-	-	-	2,3-Dihydroxybenz. Acid	94.3	85.0	67.4
COOH	OH	-	OH	-	-	β-Resorcylic Acid	97.2	93.0	79.3
COOH	OH	-	-	OH	-	Gentisic Acid	81.2	63.6	0
COOH	OH	-	-	-	OH	γ-Resorcylic Acid	69.9	44.1	0
COOH	-	OH	OH	-	-	3,4-Dihydroxybenz. Acid	107.0	100.9	81.1
COOH	-	OH	-	OH	-	3,5-Dihydroxybenz. Acid	103.1	100.8	94.5
COOH	NH_2	-	-	-	-	Anthranilic Acid	—	—	99.7
COOH	-	-	NH_2	-	-	p-Aminobenzoic Acid	—	—	100.7
COOH	OH	-	NH_2	-	-	p-Aminosalicylic Acid	—	—	91.2
$CONH_2$	OH	-	-	-	-	Salicylamide	93.9	66.3	48.1

FIG. 1. THE STRUCTURAL FORMULAE AND COMMON NAMES OF BENZOIC ACID AND SEVERAL OF ITS DERIVATIVES AND THEIR EFFECT ON THE THYROXINE (T_4)-BINDING ACTIVITY OF PREALBUMIN (TBPA) AS ASSESSED BY FILTER PAPER ELECTROPHORESIS IN TRIS-MALEATE BUFFER, pH 8.6. Under "addition to serum," values represent the proportion of a small tracer concentration of labeled T_4 bound to TBPA in the presence of inhibitor, expressed as a percentage of the comparable proportion in control specimens. Under "addition to buffer," values represent the mean of the micrograms of T_4 bound to TBPA at 386 and 450 μg of T_4 per 100 ml of serum in the presence of inhibitor, expressed as a percentage of control values. Since inhibition could be shown to be competitive, values in the presence of inhibitor are not, strictly speaking, binding capacities. At higher concentrations of T_4, additional binding of T_4 might occur, but dilution of labeled T_4 would prohibit accurate measurement.

sumption was determined on multiple occasions during the control and experimental periods. PBI or BEI values reported represent the mean of at least three determinations during each period. The T_4-binding activity of sera obtained during control and experimental periods was assessed by enriching such sera with tracer quantities of I^{131}-labeled T_4 and subjecting them to electrophoresis in filter paper in Tris-maleate buffer, as described above. Concentrations of the benzoic acid derivatives in serum were determined by a spectrophotometric technique (27). Statistical analyses were performed according to methods described by Snedecor (28).

Results

T_4 binding in serum. Figure 1 summarizes the effects of benzoic acid and 13 of its derivatives, including salicylate, on the binding of T_4 by TBPA as assessed by electrophoresis of serum in filter paper at pH 8.6. Certain of the derivatives, such as gamma-resorcylic acid and gentisic acid, when added either directly to the serum or to the buffer, were found to be potent inhibitors of T_4 binding by TBPA but did not, even at the high concentrations employed, inhibit binding of T_4 by albumin or TBG.

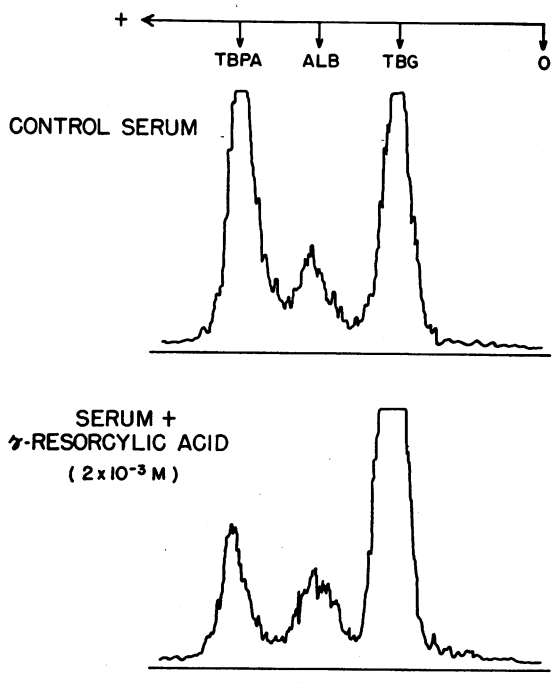


FIG. 2. THE EFFECT OF ADDING γ-RESORCYLIC ACID TO NORMAL SERUM ON THE BINDING OF A SMALL TRACER CONCENTRATION OF I^{131}-LABELED THYROXINE, AS ASSESSED BY FILTER PAPER ELECTROPHORESIS IN TRIS-MALEATE BUFFER, pH 8.6. TBPA = T_4-binding prealbumin; ALB = albumin; TBG = T_4-binding globulin.

TABLE I

*The effect of adding thyroxine (T_4) to serum on the inhibition of thyroxine-binding by TBPA induced by gamma-resorcylic acid as assessed by filter paper electrophoresis**

T_4 concentration	T_4 bound to TBPA in serum	
	Control	Gamma-resorcylic acid†
μg/100 ml	%	%
12.6	27.7	10.3
137.6	44.9	29.3
162.6	40.2	30.8
398.6	19.3	18.8
462.6	16.3	16.2

* T_4 refers to the levorotatory isomer of thyroxine. TBPA = T_4-binding prealbumin.
† At 3×10^{-3} M.

Figure 2 demonstrates a typical result obtained when the binding of an essentially endogenous concentration of T_4 in normal serum was compared with that in the same serum enriched with gamma-resorcylic acid. In the control serum, T_4 was normally distributed among TBPA, TBG, and, to a slight extent, albumin. In the serum containing gamma-resorcylic acid, in contrast, T_4 binding by TBPA was inhibited, and the T_4 displaced from TBPA was associated almost completely with TBG.

Table I demonstrates that the inhibitory activity of these derivatives, when added directly to serum, was progressively decreased and ultimately abolished by increasing concentrations of T_4. Therefore, the effect of the derivatives on the T_4-binding capacity of TBPA was assessed by adding them to the electrophoretic buffer. As shown in Figure 3, high concentrations of T_4 in the serum, when subjected to electrophoresis in Tris-maleate buffer, were bound predominantly to albumin and TBPA. The same serum was subjected to concurrent electrophoresis in buffer enriched with gamma-resorcylic acid. Here, T_4 binding by TBPA was completely suppressed, and the T_4 displaced from TBPA was bound almost entirely to albumin. In addition, the proportion of T_4 migrating to the inter-alpha globulin zone increased. Owing to the presence of a greater quantity of the derivative, the inhibition of T_4 binding by TBPA was greater when the derivatives were added to the electrophoretic buffer

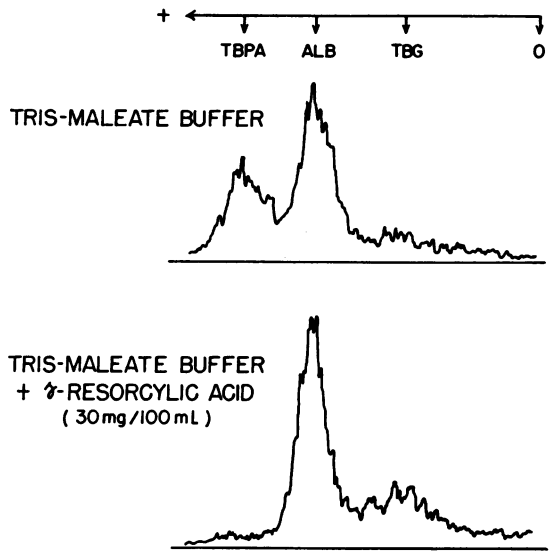


FIG. 3. THE EFFECT OF ADDING γ-RESORCYLIC ACID TO TRIS-MALEATE BUFFER, pH 8.6, ON THE THYROXINE-BINDING CAPACITY OF TBPA IN SERUM, AS ASSESSED BY FILTER PAPER ELECTROPHORESIS.

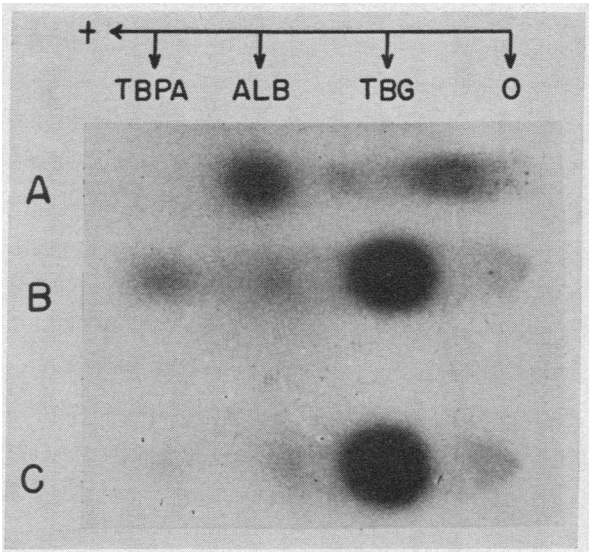


FIG. 4. RADIOAUTOGRAPH SHOWING THE EFFECT OF ADDING γ-RESORCYLIC ACID TO SERUM ON THE BINDING OF A SMALL TRACER CONCENTRATION OF I^{131}-LABELED THYROXINE, AS ASSESSED BY AGAR GEL ELECTROPHORESIS AT pH 7.4. A) Human serum albumin (1 g per 100 ml); B) control serum; C) serum enriched with γ-resorcylic acid (3×10^{-3} M).

than when they were added directly to the serum. The results obtained by the two methods, however, were qualitatively similar.

In the agar gel electrophoretic system at pH 7.4, T_4 in control serum was normally distributed among TBPA, TBG, and, to a slight extent, albumin. In the same serum to which either gamma-resorcylic acid or gentisic acid had been added, T_4 binding by TBPA was inhibited (Figure 4). Direct counting of the radioactive zones confirmed these changes and indicated that the T_4 was displaced almost quantitatively from TBPA onto TBG. No evidence was obtained that either of these derivatives interfered with T_4 binding by albumin or TBG.

As shown in Table II, gamma-resorcylic acid caused a highly significant increase in the rate of dialysis of labeled T_4 from normal serum buffered at pH 7.4. In contrast, para-hydroxybenzoic acid, which did not inhibit T_4 binding by TBPA as assessed by electrophoresis, had no effect in the dialysis system.

T_4 metabolism in vivo. Table III summarizes the results of the ten experiments carried out in the eight patients. Figure 5 presents a typical curve for the disappearance of the labeled T_4 from serum. In all the patients, an increase in the fractional rate of T_4 turnover (k) was observed during the administration of gentisic acid, gamma-resorcylic acid, or salicylamide. For the

TABLE II

*The effects of gamma-resorcylic acid and para-hydroxybenzoic acid on the rate of dialysis of I^{131}-labeled thyroxine (T_4) from whole human serum at pH 7.4**

	Rate of dialysis of PBI^{131}	p value†	T_4 bound to TBPA
	% added T_4/hour		%
Control	0.0078 ± 0.0008		27.7
Para-hydroxybenzoic acid	0.0078 ± 0.0006	>0.9	30.9
Gamma-resorcylic acid	0.0230 ± 0.0013	<0.001	10.3

* Derivatives in serum employed at a 3×10^{-3} M concentration. Values shown represent mean ± SE of five observations. PBI = protein-bound iodine.

† p values represent the probability of a chance difference from those obtained in control specimens.

TABLE III

The effects of noncalorigenic congeners of salicylate on the peripheral metabolism of thyroxine in man

Patient	Diagnosis	Experiment no.*	Period	T_4 distribution space	Fractional turnover rate†	Clearance	Protein-bound iodine	Daily disposal rate	T_4-TBPA‡	O_2 consumption
				L	*%/day*	*L/day*	*μg/100 ml*	*μg I/day*	*%*	*ml/mm*
J.W.	Normal	1	Control	8.3	8.4±0.7	0.7	6.7	46.9	34.0	
			Gentisate, 12 g/day	8.7	13.4±1.5§	1.2	4.9	58.8	20.7	
J.B.	Normal	2	Control	13.2	11.6±0.7	1.5	4.9	75.5	30.6	
			Gentisate, 12 g/day	13.2	16.2±1.8§	2.1	4.1	87.3	27.7	
G.H.	Untreated hypopituitarism	3	Control	11.0	8.8±0.6	1.0	4.4‖	42.7	26.8	147
			Gentisate, 12 g/day	12.0	11.1±0.6§	1.3	3.6‖	47.9	11.9	146
		4	Gentisate, 12 g/day	13.0	9.4±0.8	1.2	4.0‖	48.8	18.4	146
			Post-gentisate	11.3	7.0±0.8	0.8	4.4‖	34.8	28.9	
G.H.	Treated hypopituitarism	5	Control	10.9	10.7±0.3	1.2	5.7‖	65.8	36.0	175
			Gamma-resorcylate, 4 g/day	11.5	12.9±1.0§	1.5	4.9‖	73.0	26.9	178
			Post-gamma-resorcylate	8.7	11.1±0.9	1.0	6.1‖	58.9		186
R.G.	Obesity	6	Control	12.9	11.5±1.4	1.5	6.3	94.5	30.3	331
			Gamma-resorcylate, 9 g/day	13.8	13.8±0.8	1.9	5.3	100.4	22.5	342
			Post-gamma-resorcylate	10.4	12.4±1.0	1.3	6.1	78.8	31.2	346
M.F.	Untreated hypothyroidism	7	Control	12.4	7.5±0.5	0.9	1.5	14.0	27.4	156
			Gamma-resorcylate, 4 g/day	13.2	9.2±0.6	1.2	1.1	13.4	14.8	149
A.S.	Treated hypothyroidism	8	Control	10.3	9.7±1.0	1.0	5.6	56.0	23.3	175
			Gamma-resorcylate, 8 g/day	inc.	17.2±0.9§	inc.	4.1	inc.	7.4	172
		8a	Para-hydroxybenzoate, 8 g/day	11.9	9.0±0.6	1.1	5.9	63.1	26.1	
B.P.	Untreated hypothyroidism	9	Control	11.0	7.7±1.4	0.8	0.9	7.9	23.9	145
			Gamma-resorcylate, 6 g/day	11.7	8.6±0.4	1.0	0.9	9.4	10.9	147
			Para-hydroxybenzoate, 6 g/day	10.3	7.6±0.7	0.8	0.6	4.7	25.8	142
M.M.	Normal	10	Salicylamide, 8 g/day	13.6	15.2±0.7§	2.1			15.4	
			Post-salicylamide	14.7	12.5±0.4	1.8			11.7	
			Control mean	11.7	9.5	1.1	4.5	47.8	27.7	182
			Experimental mean	12.3	12.7	1.5	3.7	54.9	17.3	183
			SEMD¶	±0.26	±0.62	±0.04	±0.19	±1.83	±1.41	±2.14
			p value**	<0.10	<0.001	<0.001	<0.01	<0.01	<0.001	<0.8

* Each number indicates a separate injection of I^{131}-labeled thyroxine. In experiment 8a, a second injection was given, but control observations were not repeated.
† Values represent the mean slope ± SE as determined by the method of least squares (28).
‡ Values represent the proportion of a small tracer concentration of I^{131}-labeled thyroxine bound by TBPA in serum obtained during each treatment period.
§ Indicates significance of difference from control value in individual experiments, $p < 0.05$.
‖ Butanol-extractable iodine.
¶ SEMD = standard error of the mean difference.
** By paired *t* test (28).

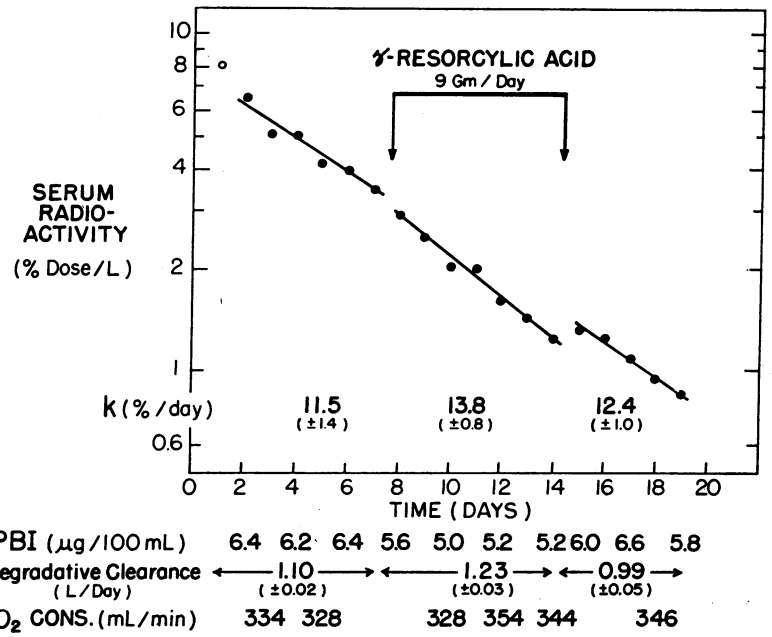


FIG. 5. THE EFFECT OF ADMINISTRATION OF γ-RESORCYLIC ACID TO PATIENT R.G. ON THE PERIPHERAL TURNOVER AND CONCENTRATION OF THYROXINE AND ON OXYGEN CONSUMPTION. PBI = protein-bound iodine; k = rate of T_4 turnover.

entire group of experiments, the average increase was 34%, and this change was highly significant statistically ($p < 0.001$). Values for k obtained in individual experiments were significantly altered by the derivative in six of ten instances. Except in the case of patient B.P., who had untreated hypothyroidism and whose PBI was abnormally low, a decrease in the PBI occurred in all experiments, and this change was highly significant statistically for the group as a whole ($p < 0.01$). Values for both the fractional turnover rate and PBI returned toward control values when the derivatives were discontinued.

As shown in Figure 5, a decrease of the radioactivity in serum occurred during the first 24 hours after the derivative was begun, whereas an increase occurred when the derivative was discontinued. In one experiment (Patient A.S.), the concentration of the radioactivity in serum fell sharply during the first 48 hours of the experimental period and then for 3 days showed little change, after which an increase in the exponential rate of disappearance occurred. Although the TDS was increased during the administration of the derivative, the long time interval required to attain a steady state precluded calculation of the

TABLE IV

The effect of gamma-resorcylic acid on the peripheral metabolism of thyroxine (T_4) in man

Patient	Period	Total T_4 clearance	Degradative clearance	Excretory clearance
		L/day	*L/day*	*L/day*
R.G.	Control	1.49	1.10 ± 0.02	0.39
	Gamma-resorcylate, 9 g/day	1.91	1.23 ± 0.03*	0.68
	Post-gamma-resorcylate	1.29	0.99 ± 0.05	0.30
B.P.	Control	0.84	0.60 ± 0.03	0.24
	Gamma-resorcylate, 6 g/day	1.01	0.72 ± 0.03†	0.29
	Para-hydroxybenzoate, 6 g/day	0.79	0.59 ± 0.03	0.20

* Indicates significance of difference from both control and post-gamma-resorcylate values, $p < 0.01$.
† Indicates significance of difference from both control and para-hydroxybenzoate values, $p < 0.02$.

TDS by the method described. In seven of the remaining nine experiments, calculated values for the TDS increased during administration of the binding inhibitors.

As a result of the changes in hormonal turnover and distribution, the daily clearance of T_4 increased in all patients. Despite the lowering of the PBI that occurred during administration of the derivative, the daily rate of hormonal disposal, representing the product of daily clearance of T_4 and PBI, increased by an average of 15%, and this change was significant ($p < 0.01$) for the group as a whole.

In one patient (A.S.), no change in the kinetics of peripheral T_4 metabolism was induced by the administration of para-hydroxybenzoic acid. In one other patient (B.P.), para-hydroxybenzoic acid failed to prevent the fractional turnover rate and PBI from returning toward control values after gamma-resorcylic acid had been withdrawn.

In the two patients (R.G. and B.P.) in whom such measurements were made, the degradative clearance of labeled T_4 increased significantly during the administration of gamma-resorcylic acid (Table IV). This value returned toward the control level when gamma-resorcylic acid was either stopped or replaced by para-hydroxybenzoic acid in a comparable dose.

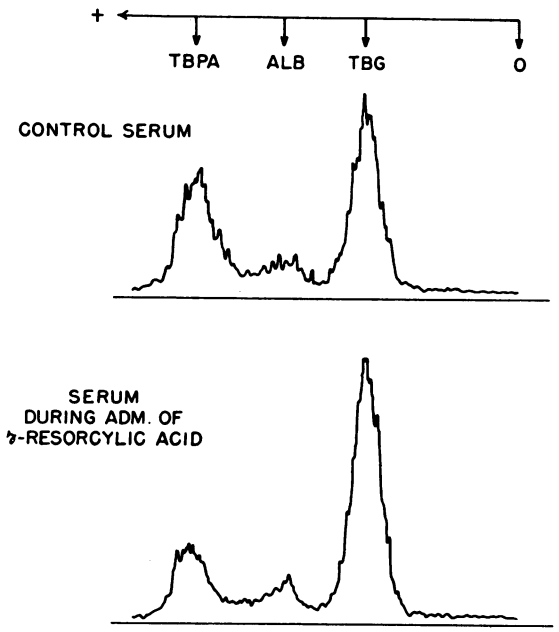


FIG. 6. CHANGES IN THE BINDING OF A SMALL TRACER CONCENTRATION OF I^{131}-LABELED THYROXINE IN SERUM DURING THE ADMINISTRATION TO PATIENT R.G. OF γ-RESORCYLIC ACID.

TABLE V

*The effect of gentisic and gamma-resorcylic acids on the deiodination of I^{131}-labeled thyroxine by slices of rat kidney cortex**

Compound	Concentration	% of control deiodination
	M	%
Gentisic acid	1×10^{-3}	22.4
	2×10^{-4}	77.4
	1×10^{-5}	86.2
Gamma-resorcylic acid	1×10^{-3}	63.4
	2×10^{-4}	96.0
	1×10^{-5}	101.0

* Values represent the proportion of thyroxine deiodinated, expressed as a percentage of the comparable value in the absence of derivative.

As shown in Figure 6, serum obtained during administration of the derivative displayed a specific inhibition of T_4 binding by TBPA when subjected to electrophoresis in Tris-maleate buffer. This change in binding was comparable to that seen when the same derivative was added directly to serum *in vitro*. No change in the binding of T_4 was seen in serum obtained during the administration of para-hydroxybenzoic acid.

In no instance did basal oxygen consumption change appreciably during the administration of the binding inhibitors.

T_4 degradation in vitro. Over a hundredfold range of concentration, encompassing those concentrations achieved *in vivo,* gamma-resorcylic acid and gentisic acid either inhibited or failed to influence the deiodination of T_4 by slices of rat kidney cortex. No stimulation of deiodination was evident (Table V).

Discussion

Increasing evidence indicates that a significant proportion of T_4 in human serum is bound by TBPA (5, 9, 10). To date, however, it has not been satisfactorily demonstrated that TBPA plays a role in regulating the peripheral metabolism of T_4 *in vivo*. Because of accompanying abnormalities, such as hypermetabolism, which follows the administration of salicylate (17), or fever (12, 13), which occurs in a variety of systemic disorders, it has not been possible to ascribe the

acceleration of T_4 turnover occurring in these states to the inhibition of T_4 binding by TBPA commonly seen in these conditions.

We found certain derivatives of benzoic acid to be specific inhibitors of T_4 binding by TBPA, both at pH 8.6 and 7.4. Indeed, two of the compounds, gentisic and gamma-resorcylic acids, proved to be more potent in this respect than salicylate itself. Both compounds have been used in the treatment of rheumatic fever and found to be free of serious side effects (29–32). In rats they are less toxic than salicylate (33). Since they also lack the calorigenic effects of salicylate (21, 22), they appeared to be ideal agents for use in assessing the effects on peripheral T_4 metabolism of inhibiting the binding of T_4 by TBPA.[7] The studies that were therefore carried out have provided what is apparently the first strong evidence that TBPA plays a significant role in regulating the peripheral metabolism of T_4 in man.

One concept of the function of the extracellular T_4-binding proteins, which appears to be true in the case of TBG, is that these proteins regulate the proportion of T_4 present in the free or unbound state and thereby limit the quantity of hormone available to the cells for metabolic action and degradation (3, 4). Within this concept, an inhibition of T_4 binding and the consequent increase in the proportion of free T_4 would permit a greater proportion of the total T_4 to become affixed to the cell. This would be manifested *in vivo* by an expansion of the TDS and, if the fractional rate of degradation of cellular hormone were essentially unchanged, by an increase in the fractional turnover of hormone in the peripheral T_4 pool. As a consequence, the total concentration of hormone in the peripheral pool would decrease until a new steady state is reached when total hormonal disposal once again equals hormonal supply. Presumably, this would occur when the total disposal of hormone has returned to normal values. If the function of TBPA is similar to that which has been ascribed to TBG, then these are the consequences that would be expected to follow the administration of gentisic acid or gamma-resorcylic acid. Such was indeed the case.

In the present studies, the administration of gentisic acid or gamma-resorcylic acid was accompanied by an abrupt lowering in the concentration of labeled T_4 in serum and by an increase in the subsequent slope of the exponential disappearance curve. As judged by conventional analysis, these changes resulted from an increase in the total volume of distribution and the fractional rate of turnover of the peripheral T_4 pool. These functions returned toward control values when the binding inhibitors were withdrawn.

Although there is evidence that the binding of T_4 limits the rapidity with which T_4 is removed from the plasma by excretory processes (34), an enhancement in the excretion of T_4 could not alone have accounted for the observed effects of the binding inhibitors, since in the two patients in whom it was measured, degradative clearance increased significantly during inhibition of T_4 binding by TBPA. Nevertheless, since the increase in degradative clearance during this time was less than the increase in total T_4 clearance, obviously the inhibitor also accelerated the excretory disposal of T_4.

An additional predicted consequence of diminished binding of T_4 would be a decrease in the PBI. We consistently observed such a decrease during the administration of the binding inhibitors, although we rarely saw values in the subnormal range. This change, however, could possibly have resulted from other mechanisms. Wolff and Austen have shown that the noncalorigenic congeners of salicylate, as well as salicylate itself, slow the rate of release of thyroidal I^{131} in rats (35); these authors have also demonstrated a comparable effect of salicylate in normal humans (35). A decreased rate of secretion of hormone could, in itself, account for a lowering of the PBI; however, several observations indicate that this was not the major factor in our studies. First, a decrease in the PBI during the administration of gamma-resorcylate, comparable to that observed in the normal patients, was seen in the patient with treated hypothyroidism. Second, in

[7] In our studies, these compounds were well tolerated by the patients and did not change the state of hydration, as judged from serial measurements of the hematocrit. In three instances, mild dyspepsia occurred, but this was readily relieved by antacids. In the dosage employed (Table III), maximal serum concentrations of 12 mg per 100 ml were attained with gentisic acid, whereas gamma-resorcylic acid was present in concentrations exceeding 20 mg per 100 ml.

all patients, a nearly maximal decline in PBI was observed within the first 24 hours of administration of the inhibitor. This decrease was greatly in excess of that which would have been expected from even a complete inhibition of T_4 secretion. Finally, in studies in patients with diffuse toxic goiter, not herein reported, gentisic acid and gamma-resorcylic acid failed to slow the disappearance of I^{131} from the thyroid but, nevertheless, caused a significant decline in the PBI (36). The small, although significant, decrease in the PBI that the derivatives induced would be consistent with previous conclusions that, although T_4 binding by TBPA may have important effects on hormonal turnover, the proportion of hormone in the serum bound by TBPA is relatively small (5).

Contrary to expectations, in most patients the daily rate of hormonal disposal was greater than the control rate during administration of the binding inhibitors. Insufficient data are available, however, to determine whether, with more prolonged administration of the inhibitors, PBI would have continued to decline and hormonal disposal would eventually have returned toward control values.

In accord with previous studies in rats (21, 22), the present experiments indicate that gamma-resorcylic and gentisic acids do not induce hypermetabolism in man. Since hormonal disposal and degradative clearance were increased by the inhibitors of TBPA, the lack of calorigenic action may indicate that the increment of cellular hormone induced by the binding inhibitors was not available for the induction of metabolic action. On the other hand, the normal individual can ingest relatively large quantities of thyroid hormone without developing hypermetabolism, and this tolerance to the hormone may explain the normal metabolic rates maintained by the patients in the present study (37).

Finally, within the limitations of extrapolating findings in animal tissues to the human situation, no evidence could be obtained that these agents directly stimulate cellular mechanisms for the deiodination of T_4. In view of all the foregoing evidence, the effects of these agents on the metabolism of T_4 in man can be ascribed only to their effect on the binding of T_4 by TBPA. The data would therefore suggest that TBPA plays an important role in regulating the peripheral metabolism of T_4 in man.

Summary

Several noncalorigenic congeners of salicylate have been found to inhibit thyroxine binding by human serum prealbumin, both at pH 8.6 and 7.4, without interfering with the binding of thyroxine by other thyroxine-binding proteins. In man, these binding inhibitors lowered the protein-bound iodine in serum and increased both the fractional and total daily turnovers of thyroxine. The changes in thyroxine metabolism occurred without hypermetabolism or systemic side effects and did not depend upon a direct stimulation of the cellular mechanisms of thyroxine degradation. Thus, the effect of these agents on thyroxine metabolism could be ascribed only to their effect on the binding of thyroxine by prealbumin. The data therefore provide strong evidence that thyroxine-binding prealbumin plays an important role in regulating the metabolism of thyroxine in man.

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Appendix

The present studies provide some information concerning the binding of small molecules by TBPA. The inhibitory potency of the derivatives of benzoic acid could be correlated with the nature and steric arrangement of substituents on the benzene ring. A hydroxyl group on carbon 2, ortho to the carboxyl group, was necessary for significant inhibitory activity. This was enhanced by additional hydroxyl substitution at carbons 5 or 6, gentisic (2,5-dihydroxybenzoic) acid and gamma-resorcylic (2, 6-dihydroxybenzoic) acid being the most potent inhibitors found. The inhibitory activity of compounds possessing an ortho-hydroxyl group was reduced by hydroxyl substitutions at carbons 3 or 4 or by amino substitutions.

The presence of an electronegative hydroxyl group ortho to the carboxyl group affects the molecule of benzoic acid in a number of ways. First, through its inductive effect, an electronegative substituent favors ionization of the carboxyl group, and this effect is greatest when the substituent is nearest or ortho to the point of attachment of the carboxyl group (38). Second, hydrogen-bond formation between the ortho-hydroxyl group and the ionized carboxyl group hinders return of the proton, thus increasing the degree of ionization of the

carboxyl group (39). Third, by invoking a charge separation in the anion, ortho- or para-substituents allow the phenyl group to participate in the resonance (38). Owing to the greater number of forms contributing to the resonance hybrid, resonance stabilization of the anion is increased. All three effects, therefore, tend to increase the acidic dissociation constants of the ortho-hydroxyl substituted derivatives over that of the parent compound. Thus, gamma-resorcylic acid, which has two ortho-hydroxyl groups, has a dissociation constant 50 times greater than that of salicylic acid and 800 times greater than that of benzoic acid (39). Thus, although it is not clear how this relationship is manifested, there appears to be a definite inverse correlation between the pK of the carboxyl group and the inhibitory potency of the compounds tested. Indeed, only the ortho-hydroxyl substituted derivatives were found to be competitive inhibitors of T_4 binding by TBPA. The high concentrations of these derivatives that were necessary to inhibit T_4 binding by TBPA and the reversal of inhibition that was achieved by relatively small concentrations of added T_4 indicate, however, that their binding constants are, by several orders of magnitude, lower than that of T_4.

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