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Studies on Thyroid Immunity. VIII Biliary Clearance[1] of Thyroxine in Thyroglobulin Immunized Rats[2] (40464)

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Our previous studies in animals immunized against thyroglobulin have shown marked alterations in the architecture of the thyroid gland, focal and diffuse lymphocytic infiltration, and localization of antibodies in the thyroid and other tissues as demonstrated by fluorescent microscopy (1, 2). Furthermore, major alterations in protein binding of thyroid hormone are also noted (3), and the rate of disappearance of thyroid hormones from the circulation is greatly reduced (4). Investigations from other laboratories have shown that the passive transfer of antiserum from an animal actively immunized against the thyroid into another species reduces plasma thyroxine (T_4) clearance in the recipient because of binding by the antibody. This avidity of immune-T_4 interaction has even been reported to be responsible for abstraction of tissue thyroxine back into the circulation (5–7). In addition, thyroglobulin has recently been shown to be normally released from the thyroid in man and in animals (8, 9); in itself thyroglobulin has also been reported to alter T_4 kinetics in mice (5) although the mechanism has not been clarified. It is possible that release of thyroglobulin from the thyroid may be accentuated in animals with induced thyroglobulin immunity due to loss of follicular integrity, and may thus influence thyroid hormone metabolism. In light of these investigations, and since liver is one of the major sites of T_4 metabolism, it was of interest to study hepatic handling of T_4 along with plasma binding and disappearance of this hormone in thyroglobulin immunized animals. The investigations showed that biliary clearance of thyroxine is markedly reduced in animals immunized with thyroglobulin; in separate investigations thyroglobulin was shown to markedly enhance biliary clearance of thyroxine in normal rats.

Methods and materials. Groups of six mature adult male rats (Holtzman) were immunized with bovine thyroglobulin (Sigma Chemical Co.) emulsified in complete Freund's adjuvant (CFA). The controls were either immunized with bovine albumin (Fraction V, Sigma Chemical Co.) in CFA or treated with CFA alone. Procedures for antigen immunization were similar to those described previously from this laboratory (3). Circulating thyroglobulin antibodies were determined by the tanned red cell (TRC) agglutination technique as noted previously (10). For albumin antibody measurement tanned red cells were coated with crystallized bovine albumin (Sigma Chemical Co.). Electrophoretic techniques used to study T_4 transport have been described in detail previously (11). Methods for studying biliary clearance of T_4 have also been described by us previously (12). Briefly, 10 μCi ^{131}I-T_4 (Amersham/Searle), or ^{125}I-T_4 in some investigations, were injected on the eve of the experiment in thyroglobulin immunized and control rats. Eighteen hours after radiothyroxine administration, common bile ducts were cannulated and bile samples collected each hour for 4 hr. Blood was drawn halfway between each bile collection. Biliary clearance was computed by dividing total radioactivity secreted in the bile

[1] We have used the phrase "biliary clearance of thyroxine" in this study instead of hepatic clearance of thyroxine because we had not identified the forms of radioactivity either in the plasma or bile. Strictly speaking, what has been measured was hepatic radioactivity clearance. However, the radioactivity cleared by the liver was all derived from exogenously administered radiothyroxine which remained more than 90% TCA precipitable in plasma during the time of the experiment; therefore, we have used the expression "biliary clearance of T_4" to describe our findings in bile after radiothyroxine administration.

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in an hour by plasma radioactivity.

Biliary clearance

$$= \frac{\text{bile c/m}}{\text{hr}} \div \frac{\text{c/m}}{\text{ml plasma}} = \text{ml/hr.}$$

Biliary clearance measurements were made in immunized rats 6–8 weeks after primary immunization. Biliary samples were subjected to thin layer chromatography (TLC) using silica gel-coated sheets. The chromatography procedure has been described in detail by us previously (13). In experiments involving passive transfer of antiserum or other materials, the test substances were injected im and 18 hr later 10 μCi ^{125}I-T_4 were administered in each animal and biliary clearance computed as noted earlier.

Whole body halftime ($T_{1/2}$) measurements of thyroxine were made using a whole body counter (Fig. 1). This technique as developed in our laboratory has not been described previously and is briefly elaborated. The lead counting chamber (id 25 cm, depth 39.4 cm) in the whole body counter, cylindrical in shape, is closed on all but the front side to facilitate animal insertion. The lead walls are 6.5 cm thick, jacketed in a 0.64 cm. steel covering, inside and outside. A lead door 7.5 cm in thickness, also jacketed in steel, is attached to the front of the chamber. The chamber is anchored to a square steel base fitted with heavy duty casters at the bottom. Along the top of the chamber are three equally spaced holes to house 2″ × 2″ NaI detectors. Around the circumference of the chamber, 120° on each side of the three detectors, and in line with the middle one, two more holes are provided to contain an additional pair of 2″ × 2″ NaI detectors. Steel clamps were fabricated and welded into the contour of the holes in the chamber to hold the detectors at any desired position. By this arrangement two types of counting geometry are possible; with all the three detector tubes on the top facing the animal vertically down (2 π geometry), or the circular geometry where the two detectors at the sides are used in combination with the center top tube. The latter arrangement was used in the present experiments. Plastic cylinders, 11.9 × 38.6 cm, were used to contain the experimental animals. These were fitted with a base and slots were made at equal distances across the length of the cylinder into which plastic slides could be inserted to prevent gross animal

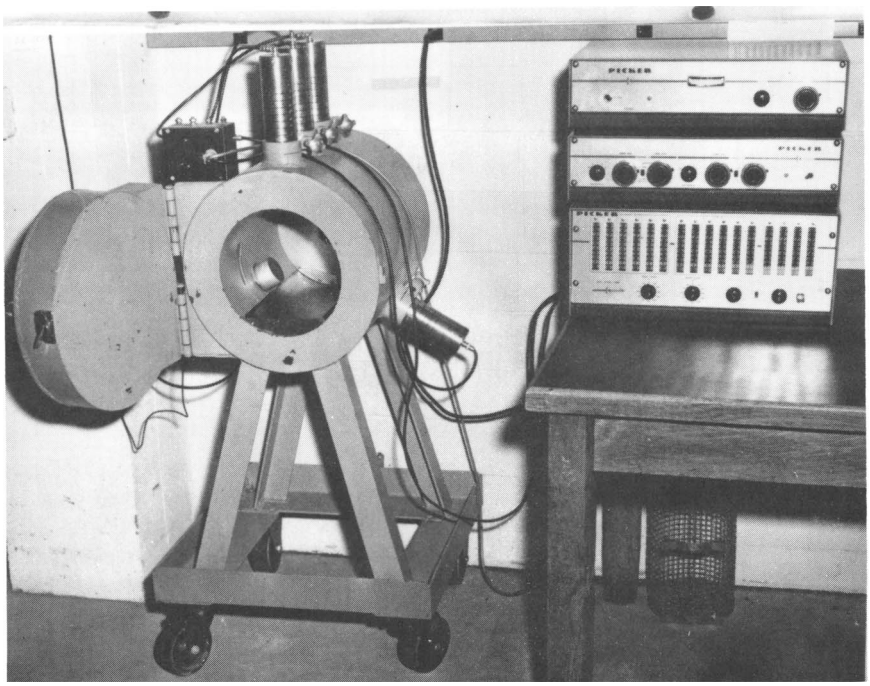

FIG. 1. Small animal whole body counter.

movements. The animals were not severely restrained in the plastic cylinder. A junction box positioned on top of the whole body counter contained preamplifiers for the detectors as well as calibrated circuitry to match the gain of the individual phototubes in the detectors; the radiation pulses were fed into a dual channel radiation analyzer and scaler (Picker Models 600057 and 600040, respectively). A switch provided in the junction box facilitated instantaneous changing of the counting geometry.

Thirty-six hours prior to $T_{1/2}$ measurements, 0.1% tapazole (methimazole, Eli Lilly, Indianapolis, IN) was added to the drinking water of each animal. ^{131}I-T_4 was administered (10 μCi iv) in each animal and the rats were counted every 2 hr for 12 hr, every 4 hr for the next 12 hr, and at 8-hr intervals thereafter for the next 48 hr. Whole body halftimes ($T_{1/2}$) of ^{131}I-T_4 were computed by least squares regression using the linear component of the disappearance curve. The $T_{1/2}$ investigations were made 1 week prior to biliary clearance measurements.

Free T_4 fraction was computed by the method of Sterling and Brenner (14).

Results. Six to eight weeks after thyroglobulin immunization all animals showed a TRC antithyroglobulin titer of at least 1/10,000 (the highest values noted were 1/50,000); albumin immunized animals displayed albumin antibody titers varying between 1/8,000 and 1/20,000. As described previously from this laboratory (10), paper electrophoresis of serum from thyroglobulin immunized rats (with added ^{125}I-T_4 tracer *in vitro*) showed the virtual localization of ^{125}I-T_4 activity at the gamma globulin region. In contrast, gamma globulin binding of ^{125}I-T_4 was not noted in adjuvant injected or albumin immunized controls.

Biliary clearance of thyroxine in animals immunized against thyroglobulin (0.26 ml/hr) was reduced four-fold ($p < .01$) in comparison to that noted in controls (1.04 ml/hr in saline controls and 1.07 ml/hr in albumin immunized rats, Table I). Quantitation of biliary radioactivity by TLC did not reveal differences in the nature of radioactivity secreted in the bile in any group (Fig. 2). The bulk of the radioactivity secreted in the bile was not thyroxine. Also, differences in biliary secretion rate were not noted between immunized and control animals.

TABLE I. BILIARY CLEARANCE OF ^{131}I-T_4,[a] WHOLE BODY HALF-TIME ($T_{1/2}$) AND SERUM TOTAL T_4 LEVELS IN RATS WITH ACTIVELY INDUCED THYROGLOBULIN IMMUNITY.

Treatment	Biliary clearance of ^{131}I-T_4 (ml/hr)	Whole body $T_{1/2}$ of ^{131}I-T_4 (hr)	Serum Total T_4 (μg/100 ml)
Saline injected control rats			
1	1.10	18.9	4.72
2	0.91	17.1	4.80
3	1.13	20.1	5.11
4	1.16	20.4	5.00
5	0.90	18.5	3.95
6	1.05	19.0	4.62
	1.04[b]	19.0	4.7
	±0.05	±0.48	0.17
Albumin immunized control rats			
7	1.18	17.9	4.20
8	0.99	18.1	5.11
9	1.10	20.8	3.95
10	1.05	18.2	4.00
11	0.95	17.0	5.00
12	1.15	20.5	4.85
	1.07	18.7	4.5
	±0.04	±0.62	±0.21
Thyroglobulin immunized rats			
13	0.29	68.6	12.9
14	0.42	60.2	16.8
15	0.28	93.6	19.2
16	0.20	68.6	14.1
17	0.15	93.6	15.9
18	0.19	100.4	16.4
	0.26	80.8	15.9
	±0.04	±6.90	±0.90

[a] This refers to radioiodine derived from administered radiothyroxine; chromatography of bile showed that bulk of the radioactivity in bile was not thyroxine.

[b] Mean ± SEM.

The thyroglobulin immunized animals showed marked prolongation of the whole body $T_{1/2}$ of ^{131}I-T_4 (80.8 hr in comparison to 19 hr in controls, Table I). The $T_{1/2}$ values in control rats as determined by the whole body counting technique were similar to those reported by other investigators who have used either whole body counting or the conventional plasma radiothyroxine measurement techniques (15, 16). Consistent with the observations of delayed disappearance of T_4 in thyroglobulin immunized rats were the estimates of percent dialyzable T_4. In control rats (saline and albumin immunized) the percent

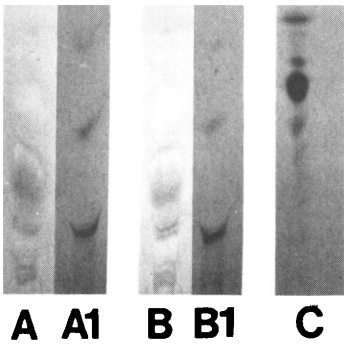

FIG. 2. Representative illustration of TLC chromatograms of bile from a control (A) and a thyroglobulin-immunized rat (B) along with their radioautographs (A1) and (B1) respectively. The intense dark band in the radioautograph at the extreme right (C) represents radiothyroxine used for reference. It is evident there was no difference in the nature of radioactivity secreted in the bile from control and thyroglobulin-immunized rats administered radiothyroxine. Little or no thyroxine was secreted in the bile of either normal or actively immunized rats.

of total ^{125}I-T_4 that was dialyzable varied between 0.045 and 0.056 with a mean of 0.052. In thyroglobulin immunized rats the percent of dialyzable T_4 was markedly reduced; the lowest and the highest values noted were 0.009 and 0.021 respectively (mean = 0.016). Although there was marked reduction in dialyzable T_4 in thyroglobulin immunized animals, they had greatly elevated T_4 levels (Table I); because of this the absolute free T_4 levels in thyroglobulin immunized animals (2.5 ng/100 ml) were essentially the same as in controls (2.4 ng/100 ml).

The primacy of effects of plasma hormone binding on biliary clearance of thyroxine was also seen in experiments involving passive transfer of immune serum from a thyroglobulin immunized rabbit (antibody N = 1.127 mg/ml), as well as other substances, into normal rats. The injection of rabbit antibovine thyroglobulin serum (which contained T_4 antibodies) promptly decreased the biliary clearance of ^{125}I-T_4 to 0.57 ml/hr, approximately half that in saline-injected animals (1.05 ml/hr). In contrast, in rats administered rabbit antihuman thyroglobulin serum or human serum with LATS (both of which sera were devoid of T_4 binding antibodies) the biliary clearance of thyroxine was essentially the same as that in controls. That the biliary secretion of T_4 is not nonspecifically influenced by various substances is also shown by the lack of effect of gelatin (a protein) and diiodotyrosine on measured clearances (Table II).

On the other hand, biliary clearance of T_4 was markedly elevated in thyroglobulin injected rats (Table II). In comparison to saline controls, the biliary clearance of T_4 in rats administered thyroglobulin was enhanced more than four-fold (4.4 ml/hr in comparison to the control value of 1.05 ml/hr). That a part of this enhancing effect of thyroglobulin on biliary clearance may be due to its T_4 content is suggested by the results noted in rats injected with T_4; the biliary clearance of ^{125}I–T_4 in T_4 administered rats was increased ~50% over the control values. The impressive effect of thyroglobulin in enhancing biliary clearance, or the pronounced effect of T_4 antibody-containing immune serum in reducing biliary clearance of thyroxine, could not be explained on the basis of differences in biliary output as may be noted in Table III. Again, as in actively immunized animals, no differences in biliary radioactivity were noted in animals used in passive transfer experiments.

TABLE II. EFFECTS OF PASSIVE TRANSFER OF ANTITHYROGLOBULIN ANTISERUM WITH OR WITHOUT T_4 BINDING ANTIBODIES, AND OTHER SUBSTANCES ON BILIARY CLEARANCE OF THYROXINE IN THE RAT.

Substances	Amounts administered	Biliary clearance (ml/hr)
Saline	2 ml	1.05 ± 0.07[a] (3)
Rabbit antibovine thyroglobulin antiserum (containing T_4 binding antibodies)	2 ml	0.57 ± 0.09 (4)
Rabbit antihuman thyroglobulin antiserum[b] (without T_4 binding antibodies)	2 ml	1.12 ± 0.04 (3)
Human serum containing LATS (but without T_4 binding antibodies)	2 ml	0.96 ± 0.03 (3)
Thyroglobulin	20 mg	4.40 ± 0.49 (4)
Gelatin	20 mg	1.16 ± 0.13 (4)
Thyroxine	.05 mg	1.50 ± 0.27 (3)
Diiodotyrosine	.50 mg	0.96 ± 0.03 (4)

Figures in parenthesis indicate the number of animals used in clearance studies.

[a] SEM.

[b] Hyland Laboratories.

TABLE III. BILIARY VOLUME AND TISSUE/PLASMA ^{125}I–T_4 RADIOACTIVITY RATIOS IN RATS ADMINISTERED ANTITHYROGLOBULIN ANTISERUM WITH OR WITHOUT T_4 BINDING ANTIBODIES, AND OTHER SUBSTANCES.

Substances	Amounts administered	Tissue radioactivity ratio c/m/g dry liver tissue/c/m/ml plasma)	Biliary volume (ml)
Rabbit antibovine thyroglobulin antiserum (containing T_4 binding antibodies)	2 ml	3.10 ±0.13[a]	0.85 ±0.10 (4)
Rabbit antihuman thyroglobulin antiserum[b] (without T_4 binding antibodies)	2 ml	4.39 ±0.39	0.88 ±0.00 (3)
Thyroglobulin	20 mg	4.98 ±0.88	0.84 ±0.07 (3)
Gelatin	20 mg	5.09 ±0.48	0.93 ±0.06 (4)

[a] SEM. Figures in parenthesis indicate the number of animals.

[b] Hyland Laboratories.

Measurement of liver tissue/plasma T_4 ratios essentially reflected plasma binding/clearance events as discussed previously. The tissue/plasma ^{125}I–T_4 ratios in animals administered substances which did not contain T_4 antibodies (4.39–5.09) were greater than that noted in animals infused with immune serum which did contain T_4 antibodies [3.10 (Table III)]. In the values shown in Table III the tissue radioactivity has not been corrected for trapped plasma radioactivity; the values if corrected would have probably brought out the differences in tissue/plasma ^{125}I–T_4 ratios more vividly between various treatment groups.

Discussion. The investigations described show that clearance from the plasma of radioactivity derived from administered radiothyroxine is markedly reduced in animals with actively induced thyroglobulin immunity. The decrease in clearance (as reflected in biliary radioactivity measurements) in thyroglobulin immunized animals was apparently specific, as shown by the absence of such changes in albumin immunized animals notwithstanding the presence of circulating albumin antibodies in titers comparable to that of thyroglobulin antibodies. Despite marked differences in biliary clearance in control and thyroglobulin immunized animals, the nature of radioactivity appearing in the bile of all animals administered radiothyroxine was the same suggesting that the form of secretion in the bile is independent of alterations in plasma binding of radiothyroxine.

The marked reduction in biliary clearance of T_4 in thyroglobulin immunized animals must be due to the binding of thyroxine by circulating antibodies. This is so because the contribution to the total observed clearance by the clearance of free T_4 is too small to affect the total; furthermore, the serum free T_4 concentrations were essentially the same in thyroglobulin immunized and control animals. On the other hand, the observations of three-fold increase in circulating T_4 in thyroglobulin immunized animals, along with the fact that the tracer dose employed was the same in all animals, would indicate a lowering of specific activity of T_4 tracer in immunized animals. Consequently, one could argue that the four-fold decrease in biliary clearance of thyroxine in immunized animals was more apparent than real since each T_4 tracer count represents three times more unlabelled T_4 in comparison to control animals. The data obtained in passive transfer experiments and certain other considerations would, however, appear to suggest that the juxtaposition of almost similar numbers (i.e. four-fold decrease in biliary clearance and three-fold increase in total T_4) is more due to chance than an actual cause-effect relation. For example, in rats (200 gm) given the same T_4 tracer dose, administration of 2 ml of human serum (T_4 = 10 μg%) failed to alter the biliary clearance of ^{125}I-T_4 despite the almost 50% increase in total intravascular T_4 pool. It may also be noted that in other published reports where similar findings have been described (i.e. increased total thyroid hormone and decreased disappearance of tracer thyroid hormone from plasma) decreases in metabolic clearance have been noted (17). Schussler *et al.* (18) recently reported a case with T_3 autoantibodies in the human with relatively high T_3, and in their study metabolic clearance rate was reduced three-fold in comparison to controls and this decrease was attributed to contraction of dis-

tribution space. The kinetics of thyroid hormone metabolism in the presence of T_4/T_3 autoantibodies in animals and in man are complex and not always straightforward. Further studies are in progress in animals with varying T_4 antibody titers to more fully understand the dynamics of biliary T_4 secretion in actively immunized animals with different T_4 pool sizes.

Further evidence that the depressive effect on biliary clearance of thyroxine in thyroglobulin immunized animals was related to antibody binding of T_4 in plasma was shown in passive transfer experiments. Biliary clearance of thyroxine was reduced in animals injected with T_4 antibody-containing antithyroglobulin serum, but not when injected with antithyroglobulin serum devoid of T_4 binding antibodies. The effects of rabbit antibovine thyroglobulin serum (containing T_4 binding antibodies) in reducing plasma clearance of T_4 is also consistent with the observations of Florsheim (5), and Solomon *et al.* (6, 7). They have described that administered rabbit antihuman thyroid immune serum in mice, not only slowed the disappearance of T_4 from the circulation, but that there was also a net mobilization of T_4 from the tissues into the blood [failure to take cognizance of this translocation of T_4 has been identified as a cause of false positive responses in McKenzie LATS bioassay procedure which depends upon measurements of blood radioactivity (6, 7, see also footnote 3)].

The effects of T_4 and thyroglobulin in enhancing biliary clearance of radiothyroxine are of particlar interest. It would appear that intrahepatocyte T_4 concentration is an important determinant in regulating biliary clearance. When T_4 alone is injected it may directly stimulate the hepatocyte to accelerate the clearance of T_4 in whatever form it is normally cleared (free and possibly some bound hormone especially to weak carrier proteins). It is also possible that during acute administration of high T_4 doses, the resulting large free T_4 pool, especially in the virtual absence of high affinity T_4 binding proteins as in the rat, might facilitate a disproportionate increase in intrahepatocyte free T_4 concentration with concomitant effects on clearance. On the other hand, the marked effect of thyroglobulin in enhancing biliary clearance may result from two additive effects. First, T_4 after its release from intracellular thyroglobulin breakdown may stimulate the hepatocyte to accelerate the clearance of T_4 in whatever form(s) T_4 is normally presented to the cell. Second, thyroglobulin by virtue of its surface binding of T_4 (19) may carry some circulating thyroxine (labeled and unlabeled) into the hepatocyte. The mode of thyroglobulin entry into the hepatocytes may be similar to other glycoproteins whose intracellular access is facilitated by the ability of the liver cells to recognize and incorporate glycoproteins (20, 21). It is also interesting to compare the effects of thyroglobulin and T_4 on biliary clearance of radiothyroxine (Table II). Twenty milligrams of thyroglobulin on complete hydrolysis could yield ~30 μg T_4 and yet it had a greater effect on biliary clearance than 50 μg T_4 alone. It is possible these differential T_4 effects on biliary clearance may result from differences in hepatocyte net T_4 uptake in the two cases. It is conceivable that the hepatocyte thyroxine concentration for a given dose of exogenously administered T_4 might be less than that attained when thyroglobulin containing an equivalent T_4 dose is injected. This is because when T_4 alone is administered the hepatocyte must compete with other plasma and tissue proteins for T_4 uptake. In the case of thyroglobulin, however, it may carry large T_4 doses directly into the cell by surface receptor recognition and internalisation of thyroglobulin.

In light of the recent studies demonstrating elevated thyroglobulin levels in certain diseases (22–25), it would be of interest to know whether elevated thyroglobulin in these states affect T_4 metabolism by diverting excessive amounts of T_4 through the biliary pathway. The effect of thyroglobulin in enhancing plasma T_4 clearance has also been noted by others (5, 26) although the specific pathways were not identified in these earlier studies. Florsheim *et al.* (5) reported that the $T_{1/2}$ of ^{125}I-T_4 in mice injected with thyroglobulin was 6.51 hours which was approximately ½ of that noted in saline injected controls (10.79 hr). The presence of thyroglobulin in test substances used in McKenzie bioassay procedure for LATS could, by enhancing biliary clearance, lower plasma thyroxine levels and may thus contribute to false negative re-

sponses.[3] The effect of thyroglobulin in inhibiting LATS response has been previously noted (5).

Among the various substances used in passive transfer experiments, the lack of effect of human serum (with its potent T_4 binding proteins TBG and TBPA) on biliary clearance seems surprising. Similar lack of effects of human serum in effecting marked reductions in T_4 clearance from circulation have also been commented upon by others (6), although some investigators have reported slight slowing of T_4 disappearance using human serum (5). However, the massive slowing of T_4 disappearance from circulation that is so regularly noted with immune serum from a rabbit actively immunized against the thyroid is not noted with human sera. The differences between human sera and immune sera from a thyroid immunized rabbit in affecting clearance may be related to quantitative differences in high affinity T_4 binding proteins present in respective sera, especially since the reported T_4 binding affinities for T_4 antibody and TBG (28, 29) are of the same order of magnitude (10^{10} M^{-1}). It may also be that the exposed chemical groupings on the T_4 antibody molecule may not fit the receptors on the liver cell as well as the TBG molecule.

Summary. Biliary clearance of radiothyroxine (^{131}I-T_4) and its whole body half-time ($T_{1/2}$) were determined in rats with actively induced thyroglobulin immunity, as well as in albumin immunized and saline injected controls. Biliary clearance in thyroglobulin immunized rats (0.26 ml/hr) was decreased four-fold in comparison to controls (1.04–1.07 ml/hr), and thin layer chromatography of radioactivity secreted in the bile showed no differences between animals. The decrease in biliary clearance in thyroglobulin immunized rats was seen coexistent with markedly reduced disappearance of ^{131}I–T_4 tracer as determined by whole body half-time measurements ($T_{1/2}$ in thyroglobulin immunized rats = 80.8 hr vs. $T_{1/2}$ of 19 hr in controls). The percent of total thyroxine that was dialyzable in thyroglobulin immunized animals (0.016) was reduced ~three-fold in comparison to control rats (0.052). The primacy of effects of plasma binding of thyroid hormone relative to its biliary clearance was further shown in passive transfer experiments where T_4 antibody-containing antithyroglobulin serum when injected into normal rats reduced biliary clearance of T_4 to half that noted in controls. On the other hand injection of thyroglobulin alone in normal rats dramatically accelerated biliary clearance of T_4 (over 400% increase in comparison to the control value of 1.05 ml/hr). The significance of these findings is briefly elaborated.

[3] It is worth noting that thyroglobulin in itself has also been shown to accentuate, and even induce LATS like response (26, 27). The results are dependent on the dosage of thyroglobulin used (27), and undoubtedly also on various other factors involved in the assay, notably on the methods used to prepare animals for LATS bioassay.

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