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# Inhibitory Action of Iodothyronines on the Thyroid. Possible Role in the Autoregulatory Mechanism

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Iodine ameliorates Graves disease symptoms and reduces goiter size. Many authors have analyzed the action of iodine on different thyroid parameters, and their results have been the subject of several recent reviews [1–3]. We have been interested, during the last decade, in the study of the mechanism of action of iodine on thyroid growth and related biochemical parameters. Our initial studies in vivo showed that the administration of potassium iodide reduces the goitrogenic action and the stimulation of protein synthesis induced by TSH, cAMP, and cGMP in mice [4]. Further in vitro studies showed that KI produces a 50% inhibition of $^{3}$H-leucine incorporation into protein. This effect is organ specific and is mediated by an intracellular and organic iodocompound, and it affects the formation of the protein backbone of thyroglobulin, but not its glycosylation. Moreover, iodothyronines were found not only to mimic the inhibitory action of KI but also to be more potent, on an equimolar basis, than that compound [5].

More recently we have examined the regulation of RNA in calf thyroid slices. KI also inhibits the incorporation of $^{3}$H-uridine and of $^{32}$P into total thyroid RNA. These actions of KI were blocked by the simultaneous addition of $KClO_4$ or MMI, again indicating that an intracellular and organic iodocompound is involved. Thyroxine mimicked the inhibitory effect of KI [6].

Later we tried to determine at which step of RNA synthesis the inhibitory action takes place. When the uptake of $^{3}$H-uridine by calf slices was measured, no significant decrease was observed under conditions where RNA specific activity was significantly decreased. Moreover, KI did not affect the $^{3}$H-inulin distribution space. A possible action at the step of nucleotide phosphorylation was then sought, and no significant change was found. As a third possibility, we explored the action of KI on the degrada-

tion rate of RNA, but no differences between controls and the KI-treated slices was found [7]. From these results we suggested that the action of KI and iodothyronines might take place at a transcriptional step, a possibility that is being explored in our laboratory. Moreover, KI inhibited the stimulatory action of cAMP and cGMP on $^{3}$H-uridine incorporation into RNA, indicating that the locus of its action is distal to cyclic nucleotide formation [7]. This finding agrees with the results obtained by Friedman et al [8] and by Delbauffe and Pavlovic-Hournac [9] when the action on thyroid protein kinase activity was studied. It should be mentioned that other groups have also shown that iodine inhibits cyclic AMP formation as well [10–12], indicating that it may have an action at more than one site.

Another important point relates to the nature of the intermediate(s) of iodine action. As mentioned before, we found that iodothyronines not only mimic the inhibitory action of KI but also are more potent, in equimolar amounts [6,7]. This finding supports the idea that these compounds may be the organic intermediates (XI compound of Dumont et al [13]), playing a role in thyroid autoregulation.

However, the possibility that iodothyronines could be dehalogenated intrathyroidally and the iodine thus originated converted to some other iodocompound had to be excluded. This hypothetical unknown substance had to be formed in very minute amounts inside the thyroid cell and therefore had to have a very high biologic activity to account for the inhibitory effects that we and others had observed.

## EXPERIMENTS

To study this hypothesis, two types of experiments were designed. In one series, the inhibitory action of $T_3$ and $T_4$ on RNA synthesis was examined in the presence of 1 mM PTU or MMI. As shown in Figures 1 and 2, the significant inhibitory action of $T_4$ and $T_3$ was not altered by the simultaneous addition of MMI or PTU. These results strongly suggested that iodothyronines have a direct action on the thyroid, manifested even when agents that block iodothyronine synthesis are employed.

To confirm our hypothesis, we studied the metabolism of labeled $T_3$ under the experimental conditions in which it is known to inhibit protein and RNA synthesis. Calf thyroid slices were incubated with $^{125}$I-$T_3$ and, after 60 minutes, homogenized and analyzed by paper chromatography. Three different solvent systems were employed: BEA: n-butanol:ethanol:0.5 N ammonium hydroxyde (5:1:2;v/v);BAW:n-butanol:acetic acid:water(138:5:6;v/v); and hexane:tertiary amyl alcohol:2 N ammonium hydroxyde(1:5:6;v/v) [14]. As shown in Figures 3, no significant dehalogenation of the $^{125}$I-$T_3$ occurred under these experimental conditions, again supporting the view that $T_3$ itself is acting on the thyroid.

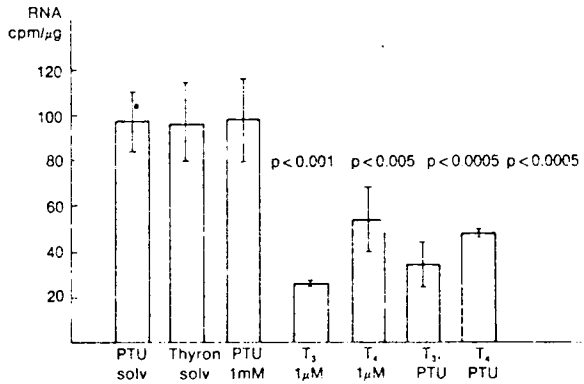


Fig. 1. Calf thyroid slices were incubated in KRB at 37°C under 95% $O_2$ + 5% $CO_2$. The solvents used to prepare PTU or the iodothyronines, were added in the control slices; PTU at 1 mM, $T_3$ at 1 µM, or PTU plus $T_3$ or $T_4$. After 30 minutes of incubation a tracer dose of $^3$H-uridine was added and the slices were pulse-labeled for 60 minutes. RNA was extracted according to the technique of Munro and Fleck, as previously described [36]. Each column represents the average of four slices ± 1 SD. Statistics were calculated according to Student's t-test.

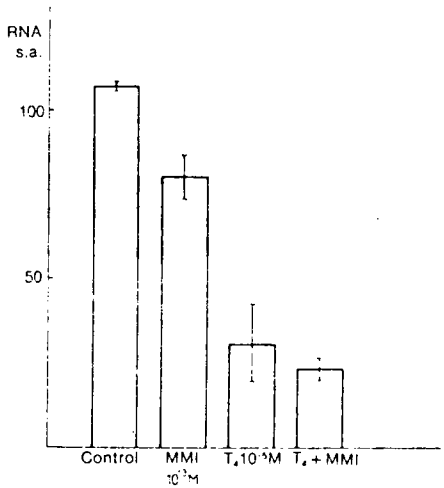


Fig. 2. Experimental conditions were the same as those described for Figure 1. MMI was used at 1 mM, and the $T_4$ concentration was $10^{-5}$ M. Each value is the average of four slices ± 1 SD. The significance of the differences was analyzed according to Student's t-test. Both groups incubated with $T_4$ or $T_4$ + MMI were significantly different ($p < 0.001$), when compared to the control slices.

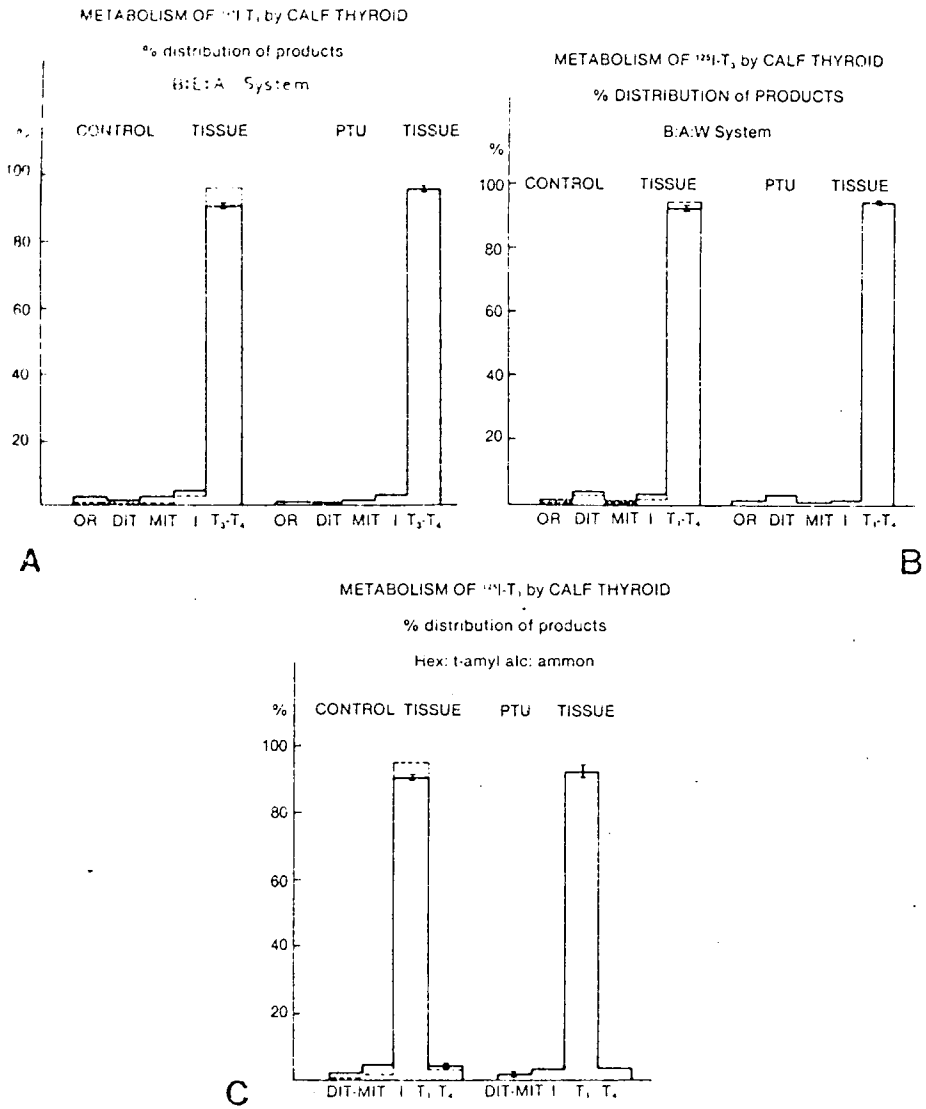


Fig. 3. Calf thyroid slices were incubated with and without 1 mM PTU under the same conditions as described for Figure 1. A tracer dose of $^{125}$I-$T_3$ was added. At the end of the incubation (60 minutes) the slices were homogenized in 1 ml of 1 mM PTU. Distribution of labeled compounds was analyzed by paper chromatography with three different solvent systems: a. ascending chromatography with BEA (n-butanol:ethanol:1 N ammonium hydroxide, 5:1:2,v v); b. BAW (n-butanol:acetic acid:water:138:5:6;v/v); and c. descending development in hexane:tertiary amyl alcohol:2 N ammonium hydroxide (1:56;v/v). The dotted line represents the results obtained with the $^{125}$I-$T_3$ before incubation. Solid lines correspond to four slices ± 1 SD from slices incubated with and without 1 mM PTU.

Later we tried to determine whether $T_3$ enters into the thyroid cell and, if so, what the characteristics of its entrance are. The T/M ratio was measured after different times of incubation at two temperatures: 37°C and 0°-4°C. The T/M values for labelled $T_3$ rose progressively with time from an initial value of 2 and reached a plateau (T/M value of 12–13) after 20–40 minutes. This process is temperature dependent, being higher at 37°C than at 0°–4°C. The addition of increasing amounts of cold $T_3$, from $10^{-9}$ M to $10^{-5}$ M, caused a progressive decrease in the T/M values. Moreover, L-$T_4$, MIT, DIT, or KI failed to alter the entrance of $T_3$ into the cell. These data suggest that a highly specific mechanism is involved in this process [15].

The influence of some other compounds was examined as well. The results indicated that the entrance of $T_3$ into the thyroid does not depend on ATP, RNA, or protein synthesis, and that it is not related to the activity of a membrane-linked ATPase. However, actinomycin D and cycloheximide produced an increase in $T_3$ entrance, and this could be due to a nonspecific effect or to inhibition of the synthesis of a repressing protein. Both cyclic AMP and cyclic GMP increased $T_3$ uptake [15].

The subcellular distribution of $^{125}$I-$T_3$ was also studied. Calf thyroid slices were incubated for 90 minutes in KRB containing the labeled hormone; with and without cold $T_3$ at $10^{-5}$ M. The percentage of subcellular distribution was compared. As shown in Table I, most of the labeled hormone was found in the 20,000 supernatant. The amount of $^{125}$I-$T_3$ in the nuclei was less than 1%, and in this purified fraction there was a highly significant decrease in the percentage of radiactivity when the slices were incubated with cold $T_3$. From this and previous results, we conclude that labeled $T_3$ is taken up by the thyroid cell in a specific manner and that there is a specific localization of the hormone in the cell nuclei.

To confirm the physiologic relevance of our findings, we studied the fate of $^{125}$I in calf thyroid slices under the same experimental conditions. Nuclei were purified from slices incubated with a tracer dose of $^{125}$I. Percentage distribution of the isotope showed that 0.5–1.5% of the $^{125}$I was localized in the nuclei. The nature of the labeled compounds was analyzed by paper chromatography. As shown in Table II, 5–6% of the radioactivity corresponded to labeled $T_3$ and approximately 3% corresponded to labeled $T_4$. These results show that, under physiologic conditions, $^{125}$I is converted into iodothyronines and that these compounds are partially localized in the nuclei.

We have also studied the relationship between the biologic activity and the chemical structure of different derivatives. Although the number of compounds tested is limited, some conclusions may be drawn. The amino acid side chain can be replaced by an acetic or propionic group without loss of its inhibitory action. However, when a formic group is placed instead, almost no inhibition of RNA synthesis occurs. Iodide can be replaced by

TABLE I. Subcellular Distribution of $^{125}I$-$T_3$ in Calf Thyroid Slices

| Subcellular fraction | Percent distribution in total homogenate | |
|---|---|---|
| | $^{125}I$-$T_3$ | $^{125}I$-$T_3$ + $10^{-5}$ M $T_3$ |
| 20,000 g supernatant | 64.0 ± 2.0 | 60.1 ± 0.7 |
| 20,000 g pellet | 20.5 ± 2.0 | 22.6 ± 1.8 |
| Nuclear supernatant (2.1 M sucrose) | 10.2 ± 0.5 | 11.6 ± 0.6 |
| First nuclear wash | 4.5 ± 0.4 | 5.1 ± 1.2 |
| Second nuclear wash | 0.6 ± 0.4 | 0.5 ± 0.2 |
| Purified nuclei | 0.21 ± 0.07 | 0.11 ± 0.02* |

*$P < 0.05$ when compared to slices incubated without cold $T_3$. Beef thyroid slices 1.5 gms were incubated in quadruplicate in KRB at 37°C for 60 minutes with a tracer dose of labeled $T_3$, with and without cold $10^{-5}$ M $T_3$. The slices were extensively washed and homogenized in 0.32 M sucrose, 1 mM $MgCl_2$, TRIS-HCl 20 mM pH 7.4, 1 mM DTT. The 800 g pellet was centrifuged at 50,000 g in 2.1 M sucrose in the same buffer containing 0.5 mM PMSF. Purified nuclei were washed with 0.25 M sucrose, the first time with 0.5% Triton and the second time without it. Each value is the average ± 1 SD.

TABLE II. $^{125}I$ Distribution in Thyroid Slices

| Experiment | % $^{125}I$ in nuclei | % Distribution in chromatography of nuclei | |
|---|---|---|---|
| | | $T_3$ | $T_4$ |
| A | 1.40 ± 0.3 | 5.6 ± 2.8 | 1.5 ± 1.2 |
| B | 0.35 ± 0.16 | 5.8 ± 0.1 | 3.0 ± 0.6 |
| C | 0.72 ± 0.18 | 4.5 ± 1.3 | 2.1 ± 0.5 |

Slices were incubated for 90 minutes under the same conditions described in Table I.

chloride in the $T_3$ and $T_4$ molecule, with full conservation of the activity; however 3,3′ diiodothyronine, or its isopropyl derivative, were devoid of action. Of great interest are the observations regarding DIMIT and DISBIT (kindly provided by Dr. G.N. Burrow, Toronto, Canada). While DIMIT had a very potent activity, DISBIT showed only marginal effects [15]. This observation fully coincides with the results of Comite et al [16], who found that DIMIT has a very potent antigoitrogenic action in the rat, whereas DISBIT is weak in this respect.

Finally, the existence of specific binding sites for $T_3$ in calf thyroid nuclei was examined. Purified nuclear preparations showed specific binding, which was linear with increasing amounts of nuclei. The binding

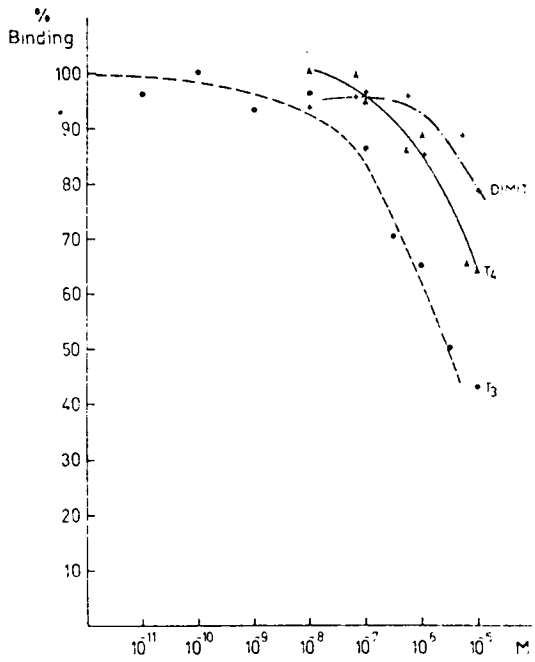


Fig. 4. Calf thyroid was homogenized in 0.32 M sucrose in TRIS-HCl 20 mM, pH7.4, 1 mM $MgCl_2$, 1 mM DTT. The 800 g pellet was resuspended in 2.1 M sucrose in the same buffer, plus 0.5 mM PMSF, and the 50,000 g pellet was washed with 0.25 M sucrose with 0.5% Triton X-100 and without it, once each. Purified nuclei were incubated in 0.25 M sucrose, 20 mM TRIS-HCl (pH7.8), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT with $^{125}$I-$T_3$ for two hours at 20°C with shaking.

reached a plateau after 90 minutes of incubation at 20°C. Addition of cold $T_3$ displaced $^{125}$I-$T_3$ from its binding sites, and $T_4$ and DIMIT had a much weaker capacity to do so (Fig. 4).

## DISCUSSION

Iodine plays an important role in the production and involution of goiter [1–3]. Previous studies from our laboratory, and from others, have shown that iodine inhibits a number of biochemical parameters [3], and many investigators have agreed that an intracellular and organic intermediate plays a role in this action. The possibility that iodothyronines might be such intermediates has been both supported and negated [see 3]. Several factors

can account for these discrepancies, namely, iodine intake, in vivo or in vitro conditions, etc. The main drawback of in vivo studies lies in the difficulty involved in differentiating an inhibition of pituitary TSH from a direct action on the thyroid. Only in vitro studies, like those presented here, should be used to show a direct action on the thyroid. Our results demonstrate that iodothyronines not only mimic iodide action but also are more potent than the halogen itself [5–7]. The findings that $T_3$ and $T_4$ exert their inhibitory action in the presence of PTU or MMI, as well as the lack of an important $T_4$ dehalogenation under the same conditions, demonstrates that these compounds have an action per se, without being converted to some other substance. There is an active uptake of $T_3$ by the thyroid cell, and the hormone is specifically localized in the nuclei. Moreover, the hormone thus localized may come either from the extracellular space or from the $T_3$ originated from the uptake of iodide intracellularly, as shown by the studies performed with $^{125}I$. This suggests that the short loop feedback mechanism in the thyroid may arise both from circulating iodothyronines and from those generated inside the cell. In this sense, it is important to note that the concentrations of $T_3$ and $T_4$ that we have used are within the intracellular physiologic range reported by several different authors [17–20]. The finding that $T_3$ is specifically localized and bound in the nuclei agrees with our hypothesis that the inhibition of RNA synthesis takes place at a transcriptional step, but further studies will be necessary before arriving at a conclusion on this point.

It has been demonstrated that the function of the thyroid gland has a certain degree of autonomy. Iodine uptake has been found to increase in response to a low iodine diet, even in hypophysectomized rats [21]. In animals fed a low-iodine diet, MTU, or PTU, thyroid weight increases well before an elevation in serum TSH occurs [22–24], again indicating that a factor intrinsic to the gland may have a relation to goiter production. In this sense, it is worth mentioning that we have reported the presence of normal or very slightly elevated TSH levels in an endemic goiter area in Argentina [25]. The demonstration that the thyroid displays an increased sensitivity to the stimulatory action of TSH in rats fed a low-iodine diet [26] provided an explanation for our findings.

Thus goiter may develop with normal concentrations of circulating TSH, both in humans and in animals, because of an increased sensitivity of the thyroid gland to its stimulating hormone. Some studies have also shown an inverse correlation between the increase in gland weight and a decrease in total organic iodine inside the thyroid [27]. These results would suggest that the decrease in intrathyroidal organic iodine would be related to the increased sensitivity of the gland.

The reverse situation has been observed under excess administration of

iodine. KI has been shown to block the goitrogenic action of TSH, cAMP, and cGMP in vivo in mice [4], as well as the incorporation of labeled amino acids into protein. Contradictory results were reported in rats. Valenta [28] demonstrated a significant decrease in protein labeling, whereas Yu et al [29] failed to confirm this finding. We have examined the effect of KI in calf thyroid slices and have shown that KI inhibits $^3$H-leucine incorporation into total and thyroglobulin protein [5], whereas Sherwin and Tong [30] found that iodine blocks the "late" stimulatory action of TSH on protein synthesis (probably related to RNA transcription). The results from our laboratory have also demonstrated that KI inhibits the in vitro synthesis of RNA in calf slices, probably at a transcriptional step [6,7].

Besides the action of iodine on biochemical steps distal to cAMP generation, an action on cyclic nucleotide formation has also been sought. Rapoport et al [12] reported that a high-iodine diet decreases thyroid response, measured as adenylate cyclase stimulation after TSH, in rats. Van Sande and Dumont [10] found a similar effect in material from dogs. This inhibitory action was confirmed both in vivo and in vitro [12, 29–32].

The inhibitory action of iodine on thyroid protein, RNA, or cAMP biosynthesis, as well as on other parameters, is mediated by an intracellular and organic iodocompound, since it is relieved by perchlorate, MMI, and PTU [5,6,10,31–33]. Moreover, the inhibitory action of iodine has been correlated with the increase in newly synthesized organic-iodine compounds in rats and mice [32,33].

As mentioned before, one of the important questions relates to the nature of the intermediates in iodine action. In vivo studies demonstrated that $T_3$ and $T_4$ pretreatment causes a decrease in different thyroid parameters in rats and mice [32,33]. However, since $T_3$ and $T_4$ cause a decrease in circulating TSH, evidence for their direct action on the thyroid is lacking. This drawback was overcomed by in vitro studies. Results from our laboratory, as well as from others, have shown that thyroid hormones can decrease protein and RNA biosynthesis as well as adenylate cyclase and protein kinase activity. These results, as well as those presented in the present paper provide strong evidence to support the idea that iodothyronines exert a direct inhibitory action on the thyroid.

A negative result has been recently reported by Goldenheim et al [34]. These authors failed to observe whether $T_3$ and $T_4$ decrease adenylate cyclase activity or TSH binding to beef thyroid membranes. However, another group of researchers reported at the same time that $T_3$ and $rT_3$ can both decrease cAMP generation in and TSH binding to purified thyroid membranes [35]. The reason for these apparent discrepancies has been explored by our group. We had the opportunity to compare the response to the inhibitory action of iodine in bovine slices obtained in Buenos Aires

(Argentina) and in Buffalo, NY. In the latter, basal RNA synthesis was not altered, whereas TSH stimulation was completely blocked. In the stock from Buenos Aires, basal RNA synthesis was usually inhibited by KI by 50–60%. When total iodine content was measured a significant difference was found. The values for the basal inhibited slices were 6.3 ± 1.3 $\mu$g iodine/mg protein, and those from slices whose TSH-stimulated RNA synthesis was inhibited by KI were 9.3 ± 2.2 $\mu$g iodine/mg protein ($P < 0.005$; average ± 1 SD from nine and eleven experiments, respectively). These results would explain the apparent failure of Goldenheim et al [34] to demonstrate an inhibition of basal adenylate cyclase activity in calves from the New England area. Moreover, they stress once more the relationship between intrathyroidal content of total and organic iodine and the gland responses to either stimuli or inhibition.

All of the above-mentioned results led us to conclude that the degree of thyroid activity, and/or its responsiveness to stimulatory or inhibitory factors, is a function of the intracellular concentration of iodothyronines ($T_3$ and, probably, $T_4$). These compounds would have specific nuclear receptors (without excluding the possibility of an action at other subcellular sites), and their modulatory signal originates either from $T_3$–$T_4$ synthetized inside the cell, or from peripheral $T_3$ taken up by the thyroid gland. This hypothesis has implications for the pathogenesis of certain types of goiter as well. As a consequence of a diffuse or localized decrease in total iodine, organic iodine, or iodothyronine concentration, the thyroid would become hypersensitive to the level of TSH that normally circulates in the blood, thus leading to either a diffuse or a nodular enlargement of the gland [see reference 3 for a more detailed discussion]. Studies are being performed in our laboratory to explore these possibilities.

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